FUNCTIONAL CHARACTERIZATION OF 100K PROTEIN OF BOVINE ADENOVIRUS TYPE 3

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ABSTRACT

Bovine adenovirus (BAdV)-3, a *Mastadenovirus* was isolated from the healthy and sick cattle (Darbyshire et al., 1965; Zhu et al., 2011). Like other adenoviruses, BAdV-3 replication is characterized by the temporally regulated expression of genes characterized by early, intermediate and late gene expression. Genus-common, non-structural protein 100K is encoded by late region L6 of BAdV-3. The objective of the present study was to characterize the BAdV-3 100K protein and identify cellular and viral proteins interacting with 100K.

Although BAdV-3 100K encoded as 850 amino acid polypeptide (Reddy et al., 1998), rabbit antisera raised against peptides representing N-terminus or C-terminus recognized a protein of 130 kDa at 12-24 hrs post infection, and proteins of 130 kDa, 100 kDa, 95 kDa and 15 kDa at 36-48 hrs post infection. The 100K appeared to be localized to the nucleus and cytoplasm of BAdV-3 infected cells. In contrast, 100K localized predominantly to cytoplasm of transfected cells. However, BAdV-3 infection of cells transfected with 100K-EYFP expressing plasmid detected fluorescent protein in nucleus of the cells suggesting that another viral protein may be required for the nuclear localization of 100K.

Using yeast two-hybrid and GST pull-down assays, 100K protein was shown to interact with BAdV-3 33K protein. These results were validated using bimolecular fluorescence complementation (BiFC) assay. Although, 100K protein interacts with 33K protein, co-expression of both proteins in transfected cells did not alter the cytoplasmic localization of 100K. Using GST-pull down assay and BiFC assay, 33K interacting region of 100K was localized to a stretch of 13 amino acids (624-637). Repeated attempts were not successful in rescuing a recombinant BAdV-3 expressing mutant 100K (containing deletion of amino acids 624-637).

The interaction of cellular protein(s) with 100K was determined by mass spectrometric analysis of immunoprecipitated 100K. Mass spectrometry of immunoprecipitate obtained by immunoprecipitating 100K protein from BAdV-3 infected cells harvested at 48 hrs post infection identified six proteins including dynein light chain (DYNLT)1. The initial identified interaction of 100K with DYNLT1 was confirmed by the yeast two-hybrid assay, co-immunoprecipitation assay and BiFC assays. Furthermore, DYNLT1 interacting domain of 100K protein of BAdV-3 was found to be located between 499-587 amino acids. Co-expression of BAdV-3 100K-EY fusion protein with myc epitope tagged DYNLT1 protein did not alter the localization of 100K-EY fusion protein.

The investigation into the differences in the subcellular localization of the 100K protein in the transfected and infected cells lead to identification of the cleavage by adenoviral protease. Subsequent analysis suggested that BAdV-3 protease cleaves 100K at two identified potential protease cleavage sites (amino acid 740-745 and 781-786) in transfected or BAdV-3 infected cells. Although protease encoded by human adenovirus (HAdV)-5 or porcine adenovirus (PAdV)-3 also cleaved BAdV-3 100K at potential identified protease cleavage sites, no such cleavage of 100K encoded by HAdV-5 or PAdV-3 could be detected in cells expressing virus specific protease. Successful isolation of recombinant BAdV-3 expressing mutant protease (substitution of alanine for glycine in potential protease cleavage site) suggested that cleavage of BAdV-3 100K by viral protease is not essential for viral replication. However, further analysis observed less virus in the supernatant of cells infected with mutant BAdV-3 compared to WT BAdV-3 suggesting a possible role for cleaved C-terminal fagment in lysis of infected cells.

Co-expression of BAdV-100K with other late viral proteins suggested that the 100K-EYFP fusion protein localized to the nucleus in cells co-expressing BAdV-3 protease-DsRed fusion

protein. Interestingly, only C-terminal cleaved fragment of 100K localizes to the nucleus in BAdV-3 protease expressing cells. Further analysis suggested that C-terminal fragment localizing to the nucleus contains a bipartite nuclear localization signal, which is recognized by importin α3. Our results suggest that the N-terminal part of 100K may be retained in the cytoplasm by interaction with Tctex1 (DYNLT1). Our study provides for the first time a plasmid co-transfection system for the study of the protease cleavage of viral proteins. Moreover, this is the first report of cleavage of any non-structural viral proteins by adenoviral protease in infected cells.

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ABBREVIATIONS USED IN THIS THESIS

Å	Angstrom
ADP	Adenovirus Death Protein
ANT	Adenine nucleotide translocase
AP	Alkaline Phosphatase
BAdV	Bovine Adenovirus
bp	Base pair
CAR	Coxsackie and adenovirus receptor
DAPI	4'-6-Diamidino-2-phenylindole
Daxx	Death domain associated protein
DMEM	Dulbeco's minimum essential medium
DYNLT1	Dynein light chain Tctex-type 1
EYFP	Enhanced Yellow Fluorescent Protein
FBS	Fetal Bovine Serum
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HAdV	Human adenovirus
HIV	Human immunodeficiency virus
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITR	Inverted terminal repeat
kb	Kilo base
kbp	Kilo base pair
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
MAb	Monoclonal antibody
MDBK	Madin-Darby bovine kidney
MEM	Minimal essential medium
MHC	Major histocompatibility complex

MLP	Major late promoter
Mnk1	MAP kinase-interacting serine/threonine-protein kinase 1
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-OH kinase
pTP	Preterminal protein
Rb	Retinoblastoma
RGD	arginine-glycine-aspartic acid
SDS	Sodium dodecyl sulfate
SMP	Skimmed Milk Powder
$TCID_{50}$	Tissue culture infectious $dose_{50}$
Tctex-1	T-complex-associated testis expressed -1
TNF	Tumor necrosis factor
TPL	Tripartite leader
VA RNA	Virus-associated RNA
WSAdV	White sturgeon adenovirus

1.0 LITERATURE REVIEW

1.1 Adenoviruses

Adenoviruses are non-enveloped, icosahedral shaped viruses, with linear double stranded DNA genome which infect wide range of vertebrates and leading to mostly non-clinical infections (Chiocca et al., 1996). The severity of the infection depends on the immunity of each individual host. Adenovirus infections are species specific and hence named after the host. Canine adenovirus-1 and -2 (Decaro et al., 2008), duck adenovirus-1 (Brash et al., 2009), fowl adenovirus-4 (Adair et al., 2008) and turkey adenovirus-3 (Pitcovski et al., 1998) are some of the economically important adenoviruses of animals. Adenoviruses served as an important experimental system in the study of the cellular mechanisms of splicing and apoptosis (Chow et al., 1977; White, 1996). Recently, there has been a great interest in the use of adenoviruses as the vectors for gene delivery and for oncotherapy (Toth and Wold, 2010; Wong et al., 2013).

1.1.1 Adenovirus taxonomy

Adenoviruses are classified under the family *Adenoviridae*, with unassigned order. Currently, family *Adenoviridae* is subdivided into five genera namely, *Mastadenovirus* (isolated from mammals), *Aviadenovirus* (isolated from birds), *Atadenovirus* (high Adenine + Thymine genome content), *Siadenovirus* (isolated from birds and reptiles) and *Ichtadenovirus* (contains a single species virus) (International Committee on Taxonomy of Viruses and King, 2012). The genome size of adenoviruses varies from 26 to 45 kbp of DNA molecule (Davison et al., 2003). There are sixteen genus common genes present in the genomes of adenoviruses which includes genes coding for DNA polymerase, terminal protein, DNA binding protein, 52K, IVa2, pIIIa, III, pVII, pX, pVI, hexon, protease, 100K, 33K, pVIII and fiber (Davison et al., 2003). With the advent

of next generation DNA sequencing technologies, classification of adenoviruses is based on DNA sequencing results. Typical adenoviral genomes can be divided into three distinct transcriptional units namely early, intermediate and late based on time of transcription after the onset of infection (Ferreira et al., 2005).

Mastadenovirus is the largest genera and has 25 species of adenoviruses of the mammals (International Committee on Taxonomy of Viruses and King, 2012). These viruses can infect humans, bovines, ovines, porcines, canines, equines, murines, bats, monkeys and shrews. Minor capsid protein pIX, core protein pV, with some proteins of E1, E3 and E4 regions are limited to *Mastadenovirus* genus only (Davison et al., 2003; Vellinga et al., 2005). Typical *Mastadenovirus* code for at least 40 proteins and human adenovirus C is the type species. Some viruses of this genus have been studied in great detail to elucidate the basic molecular host-pathogen interactions and also explored for their feasibility as viral vectors.

Aviadenovirus genus consists of 8 species of adenoviruses infecting various avian host species (International Committee on Taxonomy of Viruses and King, 2012). Based on genome sequence, *Aviadenoviruses* encode 47 to 54 open reading frames (Kajan et al., 2012). Five species were isolated from fowl (fowl adenovirus A-E), one from each falcon (falcon adenovirus A), turkey (turkey adenovirus B) and goose (goose adenovirus A) (International Committee on Taxonomy of Viruses and King, 2012). The turkey adenovirus 1 genome comprised of 45412 bp in length which makes it the longest genome of any known adenovirus (Kajan et al., 2010). The G+C % contents varies greatly among the members of this genus, turkey adenovirus 1 with 66.9 % and goose adenovirus 4 with 44.7 % is the highest and lowest respectively (Kajan et al., 2012). *Aviadenovirus* contains some homologous and some unique genes in E1 and E4 regions as compared to *Mastadenoviruses* (Grgic et al., 2011). There is complete absence of homologues of

pIX, pV, E2 and E3 regions of genes as compared to other genera of adenoviruses (Davison et al., 2003). Members of avian adenoviruses consists two fiber genes and fowl adenovirus A is a type species (Chiocca et al., 1996; International Committee on Taxonomy of Viruses and King, 2012).

Atadenovirus genus is named after the very high content of A+T in their genomes (Fields et al., 2007) and includes five species of adenoviruses (Davison et al., 2003; International Committee on Taxonomy of Viruses and King, 2012). Viruses of this genus include isolates from mammals (bovine adenovirus D, ovine adenovirus D, possum adenovirus A), a bird (duck adenovirus A) and a reptile (snake adenovirus A). Ovine adenovirus type 7 belongs to type specie ovine adenovirus D of *Atadenovirus* and does not targeted to liver and hence very promising for vector development (Bridgeman et al., 2009). Two genes p32K and RH are genus specific genes of atadenoviruses and present in E1 and E4 regions of the genome (Davison et al., 2003).

Siadenovirus genus currently consists of four species from an amphibian (frog adenovirus A – the type species) and birds (great tit adenovirus A, raptor adenovirus A, turkey adenovirus A) (International Committee on Taxonomy of Viruses and King, 2012). Viruses of this genus have the genomes of around 26 kbp, which represents the smallest adenoviral genomes known (Kovacs et al., 2010; Kovacs and Benko, 2011). Presence of sialidase gene homologue in the E1 region of these viruses gave them the name *Siadenoviruses* (Davison et al., 2003). There is complete absence of the E2 homologue of *Mastadenoviruses* in the *Siadenovirus*.

Ichtadenovirus is the fifth genus and the latest addition to the family *Adenoviridae* (International Committee on Taxonomy of Viruses and King, 2012). There is only one species sturgeon adenovirus A, which is also the type species and fish as host organism. The full length DNA sequencing of the white sturgeon adenovirus (WSAdV)-1 genome is yet to be achieved. Based on the partial DNA sequencing results of the WSAdV-1, the presence of two nucleotides

between the hexon gene and beginning of protease gene is different from other adenoviral genera (Kovacs et al., 2003). There was also the presence of two stop codons at the end of hexon genes as compared to only one in all other four recognized adenoviral genera (Kovacs et al., 2003). Phylogenetic analysis of the available DNA sequence also suggests the separate genus for WSAdV-1 (Benko et al., 2002).

1.1.2 Human adenovirus

Human adenoviruses are among the most intensively studied adenoviruses since their isolation from human adenoid tissues (Rowe et al., 1953). Generally, adenovirus does not produce clinical infections in adult healthy individuals, but can cause serious complications in immunocompromised individuals, infants and elderly. The adenoviral infections of human are limited to respiratory, enteric, ocular, renal or hepatic organs (Arnberg. 2012). Currently, there is no therapeutic anti-adenoviral drug available for the treatment of adenoviral infections (San Martin, 2012).

There are currently seven species of human adenoviruses (A through G) have been classified under the *Mastadenovirus* genus consisting at least 57 different types (International Committee on Taxonomy of Viruses and King, 2012; Walsh et al., 2011). The grouping of human adenovirus (A to G) depends on the ability of virus to agglutinate the red blood cells of different animals (human, mokney or rat), ability to produce the tumors in the experimentally infected rats and transformation of cultured cells (Fields et al., 2007; Russell, 2009). The genome organization of a typical human adenovirus includes the presence of early genes (E1 through E4), intermediate genes (pIX, IVa2) and late genes (Russell, 2009). Recently, human adenovirus isolates are grouped based on the DNA sequence of variable region of hexon, fiber or penton and other genes (Robinson et al., 2011; Walsh et al., 2011; Matsushima et al., 2012).

1.1.2.1 Virus life cycle

As viruses are obligate intracellular microorganisms, the production of the progeny virus can only takes place in the living cells. For entry into a susceptible cell, a virus needs to attach to the cell surface receptor using viral protein(s). Attachment is very specific process and consists of primary (fiber knob) and secondary (penton base) receptors attachment with cellular receptors (CAR and integrins). These specific interactions lead to the endocytosis of adenoviral particle. The acidic pH of endosomal compartment and toxic nature of the viral capsid proteins lead to release of the viral particle into the cytoplasm.

The viral particle is transported to the nucleus using microtubules and viral DNA along with some viral proteins enters the nucleus via importin receptors (Greber et al., 1997). Viral DNA associates with the histone at the active transcription sites leading to the early gene expression. Early adenoviral proteins block the apoptosis and prepare the cell for viral DNA replication (White, 2001; Giberson et al., 2012). Once the early proteins are synthesized, the viral DNA replication commences. DNA replication can be type I, which begins at both the ends of linear double stranded viral DNA through inverted terminal repeats (ITR) and type II, starts from panhandle on circular molecule from ITR (Lechner and Kelly, 1977).

Viral late gene expression is controlled by the activation of major late promoter (MLP). The MLP transcription unit is of around 29 kb and organized into five families (L1 to L5) of transcripts, based on the usage of poly (A) site (Berk, 2007). Use of different splice site lead to the transcription of around 20 species of late mRNA encoding the structural and non-structural viral proteins (Fraser et al., 1982).

The process of adenoviral assembly begins in the nucleus after the completion of the DNA replication and late gene translation. The non-structural proteins (33K, 52K and 100K) act as

chaperons or scaffolding proteins and help in the formation of capsid. Adenoviral proteins IVa2 and L1-52K proteins bind with terminal viral DNA packagaging sequences followed by insertion of viral DNA in to capsid (Ostapchuk and Hearing, 2005). The adenoviral protease cleaves at least six structural proteins before immature virions become mature virion. Mature virus release can be facilitated by the combination of action of some of the viral and host factors.

1.1.2.2 Structural proteins

Structural proteins are the components of the mature adenovirus and can be grouped into major capsid proteins (hexon, penton, fiber), minor capsid proteins (IIIa, pVI, pVIII, pIX) and core proteins (pV, pVII, Mu, terminal protein, IVa2, protease) (Russell, 2009). Recently, detailed structures of human adenovirus (HAdV)-5 have been obtained employing X-ray diffraction analysis and cryo-electron microscopy at 3.5 angstrom (Å) and 3.6 Å resolutions respectively (Liu et al., 2010; Reddy et al., 2010). The major functional details of each of the adenoviral structural proteins are given in the Table 1.2.

1.1.2.3 Non-structural proteins

The proteins that are encoded by the adenovirus and not part of the mature virion are known as non-structural proteins. Viral products encoded by E1A, E1B, E2, E3 and E4 group of early proteins along with late non-structural proteins forms the major groups of non-structural proteins of adenovirus. Adenovirus MLP controls the expression of five families of the late genes, which includes all structural proteins (except IVa2 and pIX) and many non-structural proteins (52/55K, 100K, 33K and 22K) (Berk, 2007). Details of the important non-structural proteins are provided in the Table 1.3.

1.1.3 Human adenovirus 100K protein

L4 family of late mRNA synthesizes the 100K protein, which is 805 and 807 amino acids in length in HAdV-2 and HAdV-5 respectively. The role of 100K protein in trimerization of hexon protein was suggested using temperature sensitive 100K mutant HAdV-5 cell lysate incubated with immunoprecipitated wild type 100K protein (Oosterom-Dragon and Ginsberg, 1981). Further, role of 100K protein as chaperone in the proper folding of the hexon protein was elucidated by co-expression of both the proteins in *Sf*9, an insect cell line (Hong et al., 2005). 100K protein of the HAdV-2 was found be phosphorylated mainly at serine residues in infected cells as determined by labeling experiments (Axelrod, 1978). Hexon trimerization was also assisted by 100K protein in HAdV-2 as suggested by immunoprecipation with monoclonal anti-100K antibody coupled with pulse chase experiments (Cepko and Sharp, 1982).

Role of 100K protein in the translation of late adenoviral mRNA with tripartite leader (TPL) has been studied in some detail. Initiation of translation of late and intermediate genes of adenovirus were impaired as identified by polyribosome analysis of temperature sensitive mutant (Hayes et al., 1990). Isolation of revertant virus restored the defective functions which was suggestive of the role of 100K protein in translation of TPL containing RNA (Hayes et al., 1990). Reports suggested that 100K protein of HAdV-2 interact with the TPL containing mRNA and initiates the translation of late viral mRNA using special initiation mechanism known as ribosomal shunting (Xi et al., 2004). Phosphorylation at two tyrosine residues 365 and 682 of HAdV-2 100K are essential for this function (Xi et al., 2005). 100K protein interaction with the eukaryotic initiation factor (eIF)4G initiation complex and TPL was found to be important for the process of ribosomal shunting as identified by *in vivo* RNA binding and polysomes assays (Xi et al., 2004).

Table 1.1 Role of major adenoviral factors in viral life cycle

Viral factor	Role in viral life cycle
fiber knob protein	Attachment with coxsackie and adenovirus receptor (CAR) in HAdV species A, C, D, E and F (Cupelli and Stehle, 2011; Arnberg. 2012), CD46 in species B HAdV (Gaggar et al., 2003; Marttila et al., 2005), CD80 and CD86 molecules in species B HAdV (Short et al., 2006), sialic acid in HAdV-37 (Nilsson et al., 2011).
Penton base	Integrins $\alpha_v \beta_3$ and $\alpha_v \beta 5$ interacts with RGD motif of penton base (Wickham et al., 1993) leading to activation of phosphoinositide-3-OH kinase (PI3K) (Li et al., 1998b), $p130^{CAS}$ (crk-associated substrate)(Li et al., 2000), GTP binding proteins Rac and CDC42 (Li et al., 1998a) and endocytosis of the virus particle and internalization (Cupelli and Stehle, 2011).
pVI	Breaking of endosomal compartment and release of viral particle in cytosol (Wiethoff et al., 2005; Smith et al., 2010; Scherer and Vallee, 2011).
Hexon protein	Interacts with dynein intermediate chain 2 C and dynein light intermediate chain 1 result in the viral capsid transport (Bremner et al., 2009).
pVII	Binds tightly with the adenoviral DNA and get imported to the nucleus of infected cells (Xue et al., 2005).
pVI	Displaces death domain associated protein (Daxx) from interacting with the adenoviral promoter E1A to commence the transcription of early genes (Schreiner et al., 2012). Promote the nuclear import of hexon protein (Wodrich et al., 2003).
viral DNA	Binds with the acetylated histon 3.3 suggestive of active transcription sites similar to cellular genes (Komatsu et al., 2011).
E1A protein	Conserved regions CR1 or CR2 can drive the infected cell from G_0 to S phase which creates the favorable condition for the replication of adenoviral DNA (Berk, 2005). E1A protein CR2 binds with the retinoblastoma protein and related proteins (p107 and p130) which repress the transcription factor E2F responsible for transcriptional activation of genes for progression in to S phase (Ghosh and Harter, 2003)
VA RNAI	Block the dsRNA protein kinase activation and hence allow the cell to perform the normal level of protein synthesis during the late stages of adenoviral infection (Ghadge et al., 1994).

Table 1.1 cont.

Viral factor	Role in viral life cycle
E4orf1 and E4orf4	Activate the mammalian target of rapamycin (mTOR) pathway resulting in S-phase entry of infected cell by triggering PI3 kinase and p70S6K phosphorylation respectively (O'Shea et al., 2005).
Inverted terminal repeats (ITR)	DNA replication commences from ITR (Lechner and Kelly, 1977).
Preterminal protein (pTP) and DNA polymerase	These proteins interact with the 8-17 bases of ITR viral DNA as heterodimer, which is essential for the replication of viral DNA (Temperley and Hay, 1992; Webster et al., 1997). Viral protein pTP act as the primer for DNA replication for the initiation of replication (de Jong and van der Vliet, 1999).
DNA binding protein (DBP)	Enhances the ability of the viral DNA polymerase to commence the replication of viral DNA (van Breukelen et al., 2003)
E1B-55K protein	Bind with the cellular pro-apoptotic activator protein p53 and represses the transcriptional activation of apoptotic genes (Levine. 1997; White, 2001).
E4orf6	Interact with p73, a homologue of p53 and prevents the apoptosis of infected cell (Steegenga et al., 1999).
E1B-19K	Interact with proapoptotic mitochondrial BCL-2 homologous antagonist/killer 1 protein and prevent apoptosis (Lomonosova et al., 2005).
Major late promoter	Viral proteins IVa2, pIX and 33K stimulate the MLP activation leading to expression of late genes (Lutz et al., 1997; Iftode and Flint, 2004; Ali et al., 2007).
E1B-55K and E4orf4	Selectively promote the export of viral late mRNA and blocks the cellular mRNA export from the nucleus (Blanchette et al., 2008).
100K protein	A multifunctional protein with integral role in ribosomal shunting, translation of tripartite leader (TPL) containing late viral mRNA and hexon protein synthesis (Xi et al., 2004; Xi et al., 2005; Koyuncu et al., 2012). Binds with translation initiation factor eIF4G and TPL to promote the translation of late viral genes (Xi et al., 2004). Act as chaperon in the translation, folding and trimerization of hexon protein in the cytoplasm (Hong et al., 2005).
IVa2	Bind with 22K and 52K proteins and help in viral DNA packaging in to the capsid (Ostapchuk and Hearing, 2005; Ewing et al., 2007).

Table 1.1 cont.

Viral protein	Role in viral life cycle
33K protein	A phosphoprotein act as viral splicing factor for IIIa mRNA and transcription factor for MLP (Tormanen et al., 2006; Ali et al., 2007; Tormanen Persson et al., 2012). Involved in control of late gene expression and viral DNA packaging in infected cells (Wu et al., 2013).
μ protein	Perform conformational change to adenoviral DNA and assist in the packaging of the genome in to the capsid (Russell, 2009).
Adenoviral protease	Cleaves six viral proteins (pIIIa, pVI, pVIII, pVII, pTP and pμ) and help in proteolytic maturation of virion (San Martin, 2012).
Pentone	Pentamerize in the cytoplasm and get imported to the nucleus for the capsid formation (Liu et al., 2011).
E3-11.6K	Responsible for the cell death and release of the progeny viruses (Tollefson et al., 1996).

Table 1.2 Role of major and minor adenoviral structural proteins in the viral life cycle

Viral protein	Role in viral life cycle
Hexon	Major capsid protein, which forms trimers and arranged as 12 molecules on each of 20 facets of the icosahedron (San Martin, 2012).
Penton base	Associates with four hexon trimers to form the icosahedral asymmetric unit of capsid (San Martin, 2012).
Fiber	The fiber tail interacts with the penton pentamer and the knob interacts with the CAR receptor and these interactions are essential for the attachment of the virus with the cellular surface (Cao et al., 2012).
Polypeptide IIIa	Consists of group of six-glue domain, hexon-binding domain, pVIII-binding domain and core-proximal domain (Liu et al., 2010).
pVI	Located on the inner side of the capsid under the hexon and around 360 copies per virion (Liu et al., 2010).
pVIII	Each molecule interacts with the four molecules of hexon in the capsid and hence also known as the hexon associated protein (Liu et al., 2010).
pIX	Present as trimer on facet at four positions, which act as cementing protein for the group of nine hexons (Reddy et al., 2010)
pV	Located inside the capsid beneath the major capsid proteins in mature virion.
pVII	Associated with the viral DNA as the part of nucleoprotein protecting viral nucleic acid from the action of cellular MRN (Chen et al., 2007; Karen and Hearing, 2011).
Mu	About 100 copies per virus particle attached with the viral DNA (Russell, 2009).
Terminal protein	Serve as the primer for viral DNA replication (Pronk and van der Vliet, 1993).
IVa2	Interact with the adenoviral packaging DNA sequence (Ostapchuk et al., 2005; Ostapchuk et al., 2011).
Protease	Cleaves viral proteins at consensus motif [M/I/L]-x-G-x-G or [M/I/L]-x-G-G-x and important for the maturation of virion (Webster et al., 1989; Diouri et al., 1996).

Table 1.3 Role of important non-structural proteins in the adenovirus life cycle

Viral protein	Role in viral life cycle
E1A region 289R 243R 217R 171R 55R	Produced before viral DNA replication and act as the hub and essential for the productive viral infection (Jones and Shenk, 1979; Stephens and Harlow, 1987; Ulfendahl et al., 1987; Pelka et al., 2008).
E1B region 55K 19K 156R 93R 84R	E1B-55K and 19K proteins have important roles in the viral replication and transformation of cells in culture (Takayesu et al., 1994; Sieber and Dobner, 2007).
E2 region DNA polymerase	Plays important role in viral DNA replication by interacting with the pTP for initiation (Liu et al., 2000).
E3 region 11.6K/ADP gp19K 10.4K 14.5K 6.7K 14.7K 12.5K	The E3-10.4K and 14.5K helps in immune evasion (Hilgendorf et al., 2003; Lu et al., 2009; Sharma and Andersson, 2009). E3-gp19K restricting the expression of major histocompatibility complex (MHC) class I molecules on the surface of the HAdV-2 infected cell (Fu et al., 2011). The 11.6K/ adenovirus death protein (ADP) responsible for the cell lysis and release of mature viral progeny (Tollefson et al., 1996). Protein 14.7K inhibit tumor necrosis factor (TNF) mediated cell lysis in the adenovirus-infected cells (Krajcsi et al., 1996). Protein 6.7K with 10.4K and 14.5K displaces the TNF-related apoptosis-inducing ligand receptor-2 from the adenovirus infected cell surface and prevent the apoptosis (Lichtenstein et al., 2004).
E4 region E4orf1 E4orf2 E4orf3 E4orf4 E4orf3/4 E4orf6 E4orf6/7	E4 region codes for seven proteins (Bridge and Ketner, 1989).E4orf4 responsible for induction of apoptosis transfected cells (Brestovitsky et al., 2011). E4orf1 protein was found to interact with the PI3K pathway leading to mTOR activation (O'Shea et al., 2005). E4orf6 is 294 amino acid protein with a role in degradation of p53 (Nevels et al., 2000).
33K protein	A phosphoprotein act as viral splicing factor for IIIa mRNA and transcription factor for MLP (Tormanen et al., 2006; Ali et al., 2007; Tormanen Persson et al., 2012). Involved in control of late gene expression and viral DNA packaging in infected cells (Wu et al., 2013).

Table 1.3 cont.

Viral protein	Role in viral life cycle
100K protein	A multifunctional protein with integral role in ribosomal shunting, translation of tripartite leader (TPL) containing late viral mRNA and hexon protein synthesis (Xi et al., 2004; Xi et al., 2005; Koyuncu et al., 2012). Binds with translation initiation factor eIF4G and TPL to promote the translation of late viral genes (Xi et al., 2004). Act as chaperon in the translation, folding and trimerization of hexon protein in the cytoplasm (Hong et al., 2005).
52K protein	Serotype specific viral DNA packaging (Wohl and Hearing, 2008).

The Mnk1 (MAP kinase-interacting serine/threonine-protein kinase 1) protein phosphorylates the eukaryotic initiation factor eIF4E, which in turn initiates the translation via cap-dependent scanning mechanism (Walsh and Mohr, 2011). The cap-dependent translation of cellular mRNA is inhibited by the competitive removal of Mnk1 protein from eIF4G (translation initiation factor) by the 100K protein (Cuesta et al., 2000). Inerestingly, binding of the 100K protein to RNA was not essential for the removal of the Mnk1 from the translation initiation complex (Cuesta et al., 2004).

The nuclear and cytoplasmic fractions of HAdV-2 infected HeLa cells were found to contain the 100K protein (Gambke and Deppert, 1981). Nuclear and cytoplasmic localization of Flag tagged 100K protein of HAdV-2 was reported in the transfected and infected HeLa cells (Gambke and Deppert, 1981; Cuesta et al., 2004). Presence of a functional Rev-like nuclear export signal (NES) was identified in the 100K protein ($_{383}$**L**CN**L**VSY**L**G**I**$_{392}$) of HAdV-2 and confirmed by retention of the NES deleted 100K protein in the nucleus of the transfected HeLa cells (Cuesta et al., 2004). Interestingly, transfection of C-terminally HA tagged 100K gene alone or with C-terminally myc tagged hexon gene of HAdV-5 was localized to the cytoplasm of transfected cells (Wodrich et al., 2003). Glycine-alanine-arginine (GAR) repeats (Koyuncu and Dobner, 2009) but not arginine-glycine-glycine (RGG) repeats (Cuesta et al., 2004) at the C-terminus of 100K protein were found responsible for the nuclear localization of the protein in HAdV-5 infected cells. Interestingly, the mutated 100K containing mutant NES was localized to the cytoplasm of mutant HAdV-5 (Koyuncu et al., 2012).

The RGG or GAR boxes located at the C-terminal of HAdV-5 100K protein are methylated posttranslationally at arginine residues by the action of protein arginine methyltransferase 1 (PRMT1) and are suggested to mediate the interaction of 100K with hexon protein and TPL

sequence of the viral mRNA (Koyuncu and Dobner, 2009). However, amino acids 215-420 of 100K protein of HAdV-5 interact with Hexon in GST pull-down assay (Koyuncu et al., 2012). Interestingly, the wild-type GST-100 fusion protein failed to interact with the hexon protein from the HAdV-5 infected cell lysate (Koyuncu et al., 2012).

1.1.4 Human adenovirus protease

Adenovirus protease is also referred to as adenovirus endoprotease, adenovirus endopeptidase, adenovirus proteinase and adenain in the literature. The role of the adenoviral protease in precursor polypeptide processing was suggested by the experiments with the temperature sensitive adenoviral mutants, which failed to cleave the six structural viral proteins (Weber, 1976). A highly conserved protease about 200 amino acids is encoded by all the adenoviruses studied to date (Tong, 2002; Davison et al., 2003). The protease expression was observed higher at the late phase of HAdV-2 infection and equally distributed in the cytoplamic and the nuclear fraction of HeLa cells (Webster et al., 1994). The cysteine protease inhibitors l(p-chloromercuribenzoate, iodoacetic acid, N-ethylmaleimide and 4,4'-dithiodipyridine) inhibited the bacterially expressed recombinant adenoviral protease activity to cleave HAdV-2 pVII protein (Tihanyi et al., 1993). However, based on the 2.6 Å resolution structure of HAdV-2 proteinase complex with c-terminal 11 amino acids of pVI, and the catalytic triad of H54, E71 and C122 was identified, which place it in new fifth class of cysteine proteases (Ding et al., 1996).

The pVIc (11 amino acids) and HAdV-2 DNA were biochemically identified as cofactors for the optimum protease activity (Mangel et al., 1996; Mangel et al., 1997). The presence of C-terminal fragment of adenovirus pVI ptotein or HAdV-2 DNA was found to increase protease efficiency by 355 and 6072 times, respectively as compared to protease alone (Mangel et al., 1996). For the production of infectious progeny virus, the adenoviral protease specificly cleaves at least

six major proteins at 3200 sites in total (Mangel et al., 2003). A crystal structure of adenoviral protease with a cofactor identified four large areas with positive charge which were suggested to interact with the viral DNA (Ding et al., 1996). In addition to the consensus protease cleavage sites [M/I/L]-x-G-x-G and [M/I/L]-x-G-G-x the more relaxed site specificity [M/I/L/Q]-x-G-x-G was also observed for the terminal protein of HAdV-4 (Webster et al., 1997). Sequence anaylsis of the viral protease substrate proteins suggest the absence of cysteine residues at eight amino acids on either side of the scissile bond (Ruzindana-Umunyana et al., 2002). As no anti-adenoviral drugs are available for clinical use, adenoviral protease provides the ideal target for developing therapeutic intervention (Weber, 2003).

The HAdV-2 proteins 100K, pV and hexon were found to be cleaved by adenoviral protease in an *in vitro* assay (Ruzindana-Umunyana et al., 2002) The *in vitro* cleavage of 100K protein by HAdV-2 protease was suggested to be physiologically irrelevant based on 100K protein function and activation of protease (Ruzindana-Umunyana et al., 2002).

1.1.4 Bovine adenovirus

Bovine adenovirus (BAdV) was first isolated in 1959 from respiratory secretion of a cow (Klein et al., 1959). Currently, fourteen serotypes of bovine adenovirus have been detected from the cattle samples (Lehmkuhl and Hobbs, 2008; Sibley et al., 2011) from the diseased cattle (Horner et al., 1989; Graham et al., 2005), healthy cattle (Mattson et al., 1988) or from the environment samples (Sibley et al., 2011).

1.1.4.1 Classification

Currently the BAdVs are classified under two genera *Mastadenovirus* (BAdV-1, -2, -3, -9, -10, -WIa, -WIb, -WIc) or the *Atadenovirus* (BAdV-4, -5, -6, -7, -8, -Rus) (Table 1.4) (Lehmkuhl

and Hobbs, 2008; Sibley et al., 2011). Genome length, nucleotide content and restriction enzyme analysis pattern are the criteria for placement of the BAdVs into two genera (Benko et al., 1988).

Table 1.4 Classification of bovine adenoviruses

Mastadenovirus	
Bovine adenovirus A	BAdV-1, -WIa, -WIb
Bovine adenovirus B	BAdV-3
Bovine adenovirus C	BAdV-10, -WIc
Ovine adenovirus A	BAdV-2
Human adenovirus C	BAdV-9
Atadenovirus	
Bovine adenovirus D	BAdV-4, -5, -8, -Rus
Bovine adenovirus E[a]	BAdV-6
Bovine adenovirus F[a]	BAdV-7

[a] International Committee on Taxonomy of Viruses unapproved species of bovine adenovirus (Lehmkuhl and Hobbs, 2008; Sibley et al., 2011).

1.1.4.2 Bovine adenovirus type 3

Bovine adenovirus type 3 (WBR I strain) was first isolated in 1965 from the eye swab of a healthy cow (Darbyshire et al., 1965a). BAdV-3 did produce the clinical disease in colostrum deprived calves by intranasal inoculation (Darbyshire et al., 1965b). In 2011, the BAdV-3 was isolated from the sick calves in China (Zhu et al., 2011). Tumor development was observed in hamster pups following the inoculation of BAdV-3 by various parental routes (Darbyshire. 1966). BAdV-3 uses the sialic acid receptor for the cellular entry (Li et al., 2009).

1.1.4.2.1 Genome organization

The BAdV-3 contains a double stranded DNA genome of 34,446 nucleotides with 54% G+C content (Reddy et al., 1998). The genome size of BAdV-3 is similar to HAdV-2 (35,937 bp, AC_000007.1), HAdV-5 (35,938 bp, AC_000008.1), HAdV-7 (35,514 bp, AC_000018.1) and HAdV-35 (34,794 bp, AC_000019.1) (Benson et al., 2011). Like any *Mastadenovirus*, the genome of BAdV-3 is organizied into early (E1 [E1A, E1B], E2 [E2A, E2B], E3 and E4), intermediate (IVa2, pIX), and late (L1 to L7) regions (Reddy et al., 1998). The relative position of different regions on the genome resembles to that of the prototype *Mastadenovirus* HAdV-5 except the organization of late region (Reddy et al., 1998; Davison et al., 2003). In BAdV-3, there are seven families of late genes are present as opposed to five families of late genes in HAdV-5.

Moreover, the ITR of BAdV-3 is 195 nucleotides, which is longer than the ITR HAdV-2 (102 bp) and HAdV-5 (103 bp) (Reddy et al., 1998; Davison et al., 2003).

1.1.4.2.2 Structural proteins

<u>Major Proteins</u>

The L1 region encoded penton protein is 482 amino acids long and shares identity of 44% to 64% with penton proteins of other adenoviruses (Reddy et al., 1998). The integrin interacting

motifs arginine-glycine-aspartic acid (RGD) and LDV present in penton of other adenoviruses are absent in BAdV-3 penton protein (Komoriya et al., 1991; Wickham et al., 1993; Reddy et al., 1998). Absence of the RGD motif in the penton of BAdV-3 suggests that integrin(s) may not be used as receptor for virus entry (Reddy et al., 1998). The L5 region encoding hexon protein of BAdV-3 is 910 amino acids long and shares sequence identity with hexon proteins of other adenoviruses (54.3% to 70.8%) (Reddy et al., 1998). The hexon protein monomers can be detected as 98 kDa proteins in the mature virions and empty capsids of BAdV-3 (Kulshreshtha et al., 2004). The L7 region encoding fiber protein is 976 amino acids long and shows identity of 17% to 26% with fiber proteins of other adenoviruses (Reddy et al., 1998). Three additional leader sequences present at the 5' end of HAdV-2 fiber mRNA are missing at 5' end of BAdV-3 fiber mRNA (Chow et al., 1977; Reddy et al., 1998). Like other *Mastadenoviruses*, BAdV-3 fiber protein can be devided into knob, shaft and tail regions (Reddy et al., 1998). The knob region is involved in the initial interaction of BAdV-3 with the cellular receptor (Bangari et al., 2005). The shaft region of BAdV-3 fiber is long and bent (Ruigrok et al., 1994). The tail region of BAdV-3 contains the conserved FNLVYPYKA motif and is suggested to play an important role in the interaction of fiber with the penton protein (Caillet-Boudin. 1989; Reddy et al., 1998). The BAdV-3 fiber protein is detected as 102 kDa in infected cells and contains a nuclear localization signal (the amino acids 14 to 20), which appears to be important for the viral replication in Madin-Darby bovine kidney (MDBK) cells (Wu et al., 2004). Replacement of the knob region of BAdV-3 with the knob region of HAdV-5 fiber has been helpful in altering the tropism of BAdV-3(Wu and Tikoo, 2004).

Minor Capsid Proteins

The L1 region encoded IIIa protein is 568 amino acids long and shows sequence identity of 25.2% to 57.2% with IIIa proteins of other adenoviruses (Reddy et al., 1998). Similar to the HAdV-

5 IIIa protein, the putative protease cleavage site is present in the the C-terminus of BAdV-3 IIIa (Reddy et al., 1998; San Martin, 2012).

The L4 region encoded VI protein is 263 amino acids long and shows identity of 15% to 38% with VI proteins of other adenoviruses (Reddy et al., 1998; Berk, 2007). The C-terminus of BAdV-3 VI protein is homologous to the C-terminus of HAdV-2 VI protein, which acts as the cofactor for the adenoviral protease (Reddy et al., 1998; Honkavuori et al., 2004). Similar to other other adenovirus VI, the two putative adenoviral protease cleavage sites are also present in the BAdV-3 pVI protein (Reddy et al., 1998).

The L6 encoded VIII protein is 216 amino acids long and shares 19% to 56.5% sequence identity with VIII proteins of other adenoviruses (Reddy et al., 1998). BAdV-3 pVIII possesses two putative adenoviral protease cleavage sites at amino acid position 108 and 143 (Reddy et al., 1998). Interestingly, only two of the three reported protease cleavage sites in HAdV-5 pVIII protein (San Martin, 2012) are present in BAdV-3 VII.

The intermediate region encoded pIX protein is 125 amino acids long and shares 16.5% to 27.2% with pIX proteins of other adenoviruses (Reddy et al., 1998; Reddy et al., 1999a). The pIX protein is detected as 14 kDa protein in purified virions and in virus infected cells (Zakhartchouk et al., 2004) The C-terminus of pIX is exposed on the surface of the virion, which is utilized to incorporate heterologous proteins (Zakhartchouk et al., 2004).

Core proteins

The L1 region encoding the VII protein is 171 amino acid long and shares 29.7% to 53.2% identity with VII proteins of other adenoviruses (Reddy et al., 1998). The BAdV-3 VII protein contains a mitochondrial localization signal (amino acid 1-54), localizes to mitochondria, and

modulates ATP synthesis, mitochondrial Ca^{2+} and mitochondrial membrane potential (Anand, 2011).

The L2 region encoded V protein is 410 amino acids and shares 28.3% to 40.9% identity with V proteins of other adenoviruses (Reddy et al., 1998). The BAdV-3 V is detected at 36 hrs post infection as a 56 kDa protein in infected cells (Kulshreshtha and Tikoo, 2008).

The L3 region encoded X protein is 80 amino acids long (Anderson et al., 1989; Reddy et al., 1998) and contains two putative protease cleavage sites (Reddy et al., 1998). The L5 region encoded protease is 204 amino acid long and shares 30.3% to 65.7% identity with proteases of other adenoviruses (Reddy et al., 1998; Weber, 2007).

The E2B region encoded terminal protein (TP) is 649 amino acids long and has shares 24% to 59.9% sequence identity with TP of other adenoviruses (Reddy et al., 1998).

The E2B encoded IVa2 protein is 376 amino acids long and shares 29% to 69% identity with IVa2 proteins of other adenoviruses (Reddy et al., 1998).

1.1.4.2.3 Non-structural proteins

E1 region: The E1 region expressed E1A is located between map units 0.8-10.5 and produces proteins of 211, 115 and 100 amino acids from alternatively spliced mRNAs. The E1A proteins are detected as 43, 57 and 65 kDa proteins at 12-48 hrs post infection in virus infected cells (Reddy et al., 1999b). The E1A protein (s) appear essential for replication of BAdV-3 (Zhou et al., 2001). The E1 expressed E1B region maps to 4.2-10.5 map units and produces proteins of 157 and 420 amino acids from two overlapping mRNAs (Reddy et al., 1999). The E1B proteins are detected as 19 kDa and 48 kDa proteins from 6-48 hrs post infection in virus infected cells (Reddy et al., 1999b). While 19 kDa protein appears non-essential for BAdV-3 replication in fetal bovine retina cells, it appears essential for BAdV-3 replication in bovine fibroblast cells (Zhou et al., 2001).

Unlike HAdV-2, the E1A and E1B transcripts of BAdV-3 shares the common 3' terminus (Berk and Sharp, 1978; Reddy et al., 1999a). Interestingly, a replication-competent BAdV-3 could be isolated by deleting the DNA sequence between left ITR and start of E1A region suggesting that ITR may act as promoter for E1A transcrion (Xing and Tikoo, 2006).

E3 region: The E3 region is located between map units 75 to 83 and produces proteins of 284, 121, 86 and 82 amino acids from spliced mRNAs (Idamakanti et al., 1999). The E3 proteins are detected as glycoproteins of 48, 67 and 125 kDa and a protein of 14.7 kDa (Idamakanti et al., 1999). The 67 and 125 kDa proteins appear to be post translationally modified forms of the 48 kDa protein (Idamakanti et al., 1999). The E3 proteins are not essential for replication of BAdV-3 *in vitro* (Zakhartchouk et al., 1998) or *in vivo* (Zakhartchouk et al., 1999). However, BAdV-3 E314.7 kDa protein inhibits cytolysis of mouse cells by tumor necrosis factor (Zakhartchouk et al., 2001).

E4 region: The E4 region is located between map units 98.6 to 89.9 (Lee et al., 1998). The E4 region transcribes seven mRNAs which has the potential to encode unique proteins of 69, 143, 143, 219 268 animo acids (Baxi et al., 1999). Mutational analysis of E4 region suggests that none of the individual proteins appear essential for replication of BAdV-3 (Baxi et al., 2001).

Late region: The L2 encoded 52K protein is 370 amino acids long (Paterson, 2010) and shares identity 21.4% to 61.6% with 52K protein of other adenoviruses (Reddy et al., 1998). The 52K protein contains a bipartite nuclear localization signal and utilizes the nuclear import receptor importin α3 to localize in the nucleus of the transfected and virus infected cells (Paterson et al., 2012). The 52K protein interacts with BAdV-3 encoded pVII and cellular protein NFkB-binding protein (Paterson, 2010). Co–expression of 52K and NFBP results in redistribution of NFBP from nucleolus to other parts of nucleus (Paterson, 2010).

The L6 region encodes three proteins namely 33K, 22K and 100K. The 33K protein, a spliced form of 33K mRNA is 279 amino acids long. The 33 K protein is detected as two major proteins of 42 kDa and 22 kDa and five minor proteins of 39 KdA, 35 kDa, 29 kDa 25 KdA and 19 kDa in virus infected cells (Kulshreshtha, 2009). The 33K localizes to nucleus and activates transcription from the major late promoter (Kulshreshtha, 2009). The highly conserved C-terminus of 33K appears to contain nuclear localization signals and major late preomoter DNA binding domains (Kulshreshtha, 2009). The 33K protein have been shown to interact with BAdV-3 protein pV and 100K (Kulshreshtha and Tikoo, 2008), and cellular protein bovine presenilin-1-associated protein (BoPSAP) (Kulshreshtha, 2009) using coimmunoprecipitation assay.

The 22K, an unspliced form of 33K mRNA is 274 amino acids in length. The 22K protein is detected as 41 kDa, 39 kDa and 37 kDa proteins in infected cells (Kulshreshtha, 2009). The 33K and 22K proteins share N-terminal 138 amino acids, which appear to be essential for replication of BAdV-3 including capsid assembly and/or capsid DNA encapsidation (Kulshreshtha et al., 2004)

The L6 encoded 100K is 850 amino acids long and shares 27.6% to 52.1% identity with100K proteins of other adenoviruses (Reddy et al., 1998). Like other adenoviruses, the C-terminus of 100K overlapps with 33K/22K proteins

The U exon protein (UXP) is 55 amino acids and is encoded by l strand of BAdV-3, which overlaps with the fiber gene and E3 region (Reddy et al., 1998; Ying et al., 2010). The UXP of HAdV-5 can be detected at 24 kDa from the infected cell lysate at 18 to 48 h post infection suggestive of mRNA splicing and localizes to the nucleus of infected A549 cells (Reddy et al., 1998).

1.2 Protein-protein interactions

Protein-protein interactions are the most important processes responsible for many viral/cellular functions at molecular level, which can involve two or more proteins (Sardiu and Washburn, 2011). Protein-protein interactions determine the final outcome of the host-pathogen relationship (Franzosa and Xia, 2011). Knowledge of the protein-protein interactions at molecular level can help in judging the disease outcome and formulating the strategy for treatment or prevention of the pathogenic infection of human and animals (Corcoran and Doyle, 2004; Li et al., 2005; Miura and Holmes, 2009). Currently, the virus-host protein interactions are one of the major areas of virus research.

1.2.1 Adenovirus-host protein interactions

As in other viruses, adenovirus proteins interact with the host proteins throughout the viral replication cycle and help in making favorable cellular environment for production of progeny virions. Study of the adenoviral-host protein interactions had lead to the understanding of cellular molecular processes including the discovery of Coxsackievirus and adenovirus receptor (CAR). Most of the host-pathogen interaction data available are the results of the studies on HAdV-2 and -5. Adenoviral proteins vary in terms of the number of cellular proteins they interact with, where some proteins have many cellular protein partners identified and some have few or none. Protein interactions of viral and cellular proteins, which have been discussed in previous sections, have been omitted to avoid repetition.

1.2.1.1 Structural proteins

The protein-protein interactions between viral structural proteins and cellular proteins are listed in the Table 1.5.

1.2.1.2 Non-structural proteins

The protein-protein interactions between viral non-structural proteins and cellular proteins are listed in the Table 1.6.

1.2.2 Adenoviral protein-protein interactions

The protein-protein interactions between viral proteins during the adenoviral infection process are listed in the Table 1.7.

Table 1.5 Protein interactions involving structural proteins during the adenovirus infection

Viral protein	Cellular protein	Functional significance
Hexon	Scavenger receptor II	Facilitate the infection of Kupffer cells with HAdV-5 (Khare et al., 2012)
Hexon	blood clotting factor X	Recognition by innate immune system of the invading HAdV-5 (Doronin et al., 2012)
Penton	Bcl-2-associated athanogene 3	Important for the cellular entry and progeny production in HAdV-2 infected cells (Gout et al., 2010)
Fiber	GD1a ganglioside	Infection of corneal cells with HAdV-37 (Nilsson et al., 2011)
pVI	Importin α/β	Nuclear import of pVI (Wodrich et al., 2003)
pIX	Unknown	Activation of TATA containing cellular and viral promoters (Lutz et al., 1997)
pV	Nucleolin and B23	Displaces nuclear proteins during the HAdV-2 infection process (Matthews, 2001)
pVII	Template-activating factor I α (SET)	Forms the complex with the viral chromatin in HAdV-5 infected cells (Xue et al., 2005)
pVII	Phosphoprotein 32 (pp32)	Forms the complex with the viral chromatin in HAdV-5 infected cells (Xue et al., 2005)
pTP	Octamer transcription factor 1 (Oct-1)	Stimulate DNA replication (Coenjaerts et al., 1994)
pTP	Nuclear factor I (NFI)	Positively influence the viral DNA replication (Coenjaerts et al., 1994)

Table 1.6 Protein interactions involving non-structural proteins during the adenovirus infection

Viral protein	Cellular protein	Functional significance
E1A protein (289R)	Transcription factor E2F/DP-1 protein complex	induction of S-phase in adenovirus infected cells (Pelka et al., 2011)
E1A protein	Forkhead box protein (FOX) K1/K2	Supress cell proliferation and transformation in HAdV-5 infected cells (Komorek et al., 2010)
E1A proteins (289R and 243R)	Yin and yang (YY1)	Relieving the repression by YY1 protein in infected cells (Lewis et al., 1995)
E1A proteins (289R and 243R)	BS69	BS69 suppresses the E1A activated transcription in transfected cells (Hateboer et al., 1995)
E1A protein	Mediator of RNA polymerase II transcription subunit 23 (mSur2)	Plays an important role in the viral replication of mouse adenovirus type 1 (MAdV-1) (Fang et al., 2004; Fang and Spindler, 2005)
E1A protein	Histone acetyltransferase p300	Entry of infected cell into S-phase (Eckner et al., 1994)
E1A protein	CREB binding protein (CBP)	Suppression of myogenesis in HAdV-5 infected cells (Reid et al., 1998; O'Connor et al., 1999)
E1A protein	Dual-specificity Yak1-related kinases (DYRK)	Cell cycle and tumorigenicity regulation (Zhang et al., 2001)
E1A protein	Cyclin-dependent kinase inhibitor 1 (p21)	Regulation of apoptosis in the transfected cells (Chattopadhyay et al., 2001)
E1A protein	Transcription factor p65	Inhibition of transcriptional activation in HAdV-12 infected cells (Jiao et al., 2010)

Table 1.6 cont.

Viral protein	Cellular protein	Functional significance
E1A protein (289R)	Histone acetyltransferase GCN5	Negative regulation of transactivation in the transfection and knock-down assays (Ablack et al., 2012)
E1A protein	PML nuclear bodies (PML-II)	Transactivation of viral promoter of HAdV-5 (Berscheminski et al., 2013)
E1B-55K protein	PCAF protein	Resulting in the inhibition of p53 acetylation affecting the cellular transformation by HAdV-12 (Liu et al., 2000)
E1B-55K protein	Wilms tumor protein (WT1)	Inhibition of cellular apoptosis in transfected osteosarcoma cells (Maheswaran et al., 1998)
E1B-55K protein	p53 protein	Functional inactivation of tumore suppressor p53 (Maheswaran et al., 1998)
E1B-55K protein	Daxx	Inhibition of p53 mediated transactivation of Bax promoter sequences (Zhao et al., 2003)
E1B-55K protein	mSin3A	Co-repressor mSin3A protein interaction is not required for the p53 mediated transcriptional repression for HAdV-12 (Zhao et al., 2007)
E1B-156R protein	Daxx and p53 proteins	Unknown functional significance in the HAdV-5 infected cells (Sieber and Dobner, 2007)
E3-14.7K protein	NF-kappa-B essential modulator (FIP-3)	Prevents the cell apoptosis by FIP-3 in co-transfected cells (Li et al., 1999)
E3-ADP	Mitotic arrest deficient 2-like protein 2 (MAD2B) protein	Important role in the cell lysis and mature virion release in HAdV-5 infected cells (Ying and Wold, 2003)
E4orf1 protein	Disks large 1 (Dlg1)	May have role in tumorigenesis in the HAdV-9 infected cells (Lee et al., 1997)
E4orf1 protein	Multiple PDZ domain protein (MUPP1)	HAdV-9 infected cell transformation (Lee et al., 2000)

Table 1.6 cont.

Viral protein	Cellular protein	Functional significance
E4orf4 protein	Yeast nucleoside diphosphatase 1 (Ynd1)	Apoptosis of the transformed yeast cells (Mittelman et al., 2010)
33K protein	DNA-dependent protein kinase (DNA-PK)	DNA-PK negatively regulates the early to late L1 alternative splicing (Tormanen Persson et al., 2012)
33K protein	Protein kinase A (PKA)	PKA activates early to late L1 alternative splicing (Tormanen Persson et al., 2012)

Table 1.7 Protein interactions involving viral proteins during the adenovirus infection

Viral protein	Viral protein	Functional significance
L1-52/55K protein	IVa2 protein	Transcription from MLP and viral assembly formation in HAdV-5 (Gustin et al., 1996)
E1B-55K protein	E1B-55K protein	Homodimer formation important for association with the p53 protein in HAdV-5 infected cells (Morawska-Onyszczuk et al., 2010)
E4orf6 protein	E1B-55K protein	HAdV-5 E1B-55K protein get translocated from cytoplasm to the nucleus in the presence of HAdV-5 E4orf6 protein in cotransfected cells (Goodrum et al., 1996)
22K protein	22K protein	The exact significance is unknown (Yang and Maluf, 2010)
E1A protein	pVII	Association is necessary for the recruitment of E1A protein to the transcription sites (Johnson et al., 2004)
IVa2 protein	33K	Forms a trimeric complex with DBP to play an important role in viral genome packaging (Ahi et al., 2013)

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Rationale for hypothesis

Adenoviruses have provided a model system for the study of the host-pathogen interactions since 1953 (Rowe et al., 1953). Most adenoviruses are non-pathogenic in nature but economically important infections like infectious canine hepatitis, kennel cough, egg drop syndrome, hydropericardium syndrome and haemorrhagic enteritis results from them. Like other adenoviruses, BAdV-3 is non-pathogenic and grows to high titers. Though molecular characterization of BAdV-3 has demonstrated similarities with other members of *Mastadenoviruses* (Baxi et al., 1998; Reddy et al., 1998; Reddy et al., 1999), recent studies identified distinct features of BAdV-3 (Patel and Tikoo, 2006; Xing and Tikoo, 2006; Kulshreshtha and Tikoo, 2008). Since the production of progeny virus involve both protein-DNA and protein-protein interactions, many structural proteins, non-structural proteins and cellular proteins appear to be involved in these interactions (Russell, 2009). Determining the importance of these interactions would help not only in understanding the biology of BAdV-3 but may also help in designing an efficient vaccine delivery vehicle (Mittal et al., 1999; Babiuk and Tikoo, 2000; Reddy et al., 2000; Wu and Tikoo, 2004; Xing and Tikoo, 2006; Zakhartchouk et al., 2007).

One of sixteen genus-common genes is the non-structural protein named 100K found in all the adenoviruses reported so far (Davison et al., 2003). Adenovirus 100K protein appears essential for the replication of the HAdV-5 (Hodges et al., 2001). Adenovirus 100K protein is involved in the trimerization of adenoviral hexon protein (Morin and Boulanger, 1986; Hong et al., 2005) and in translation of late viral mRNA using special initiation mechanism known as ribosomal shunting (Xi et al., 2004). Although, adenoviral 100K protein has been shown to interact with the cellular translation initiation factor eIF4G, (Cuesta et al., 2000), adenoviral hexon protein (Hong et al.,

2005) and 33K protein, many functions performed by 100K may require interaction(s) with other cellular or viral proteins.

2.2 Hypothesis

I hypothesize that BAdV-3 100K protein interacts with other viral and cellular proteins during the BAdV-3 infection of bovine cells.

2.3 Objectives

The work presented in this thesis is directed to characterize the 100K protein and its interactions in BAdV-3. The following specific objectives were designed to test the hypothesis.

i. To characterize the expression and localization pattern of the 100K protein in BAdV-3 infected and plasmid DNA transfected cells.
ii. To identify the cellular and other viral protein partners interacting with the 100K protein of BAdV-3.
iii. To attempt the identification of biological significance of observed protein-protein interactions.

3.0 INTERACTION BETWEEN 100K AND 33K PROTEINS OF BOVINE ADENOVIRUS-3

3.1 Introduction

Adenoviruses are non-enveloped, icosahedral, DNA viruses of the vertebrates with a genome size of 26-45 kbp (Davison et al., 2003; San Martin, 2012). Bovine adenovirus (BAdV)-3 belongs to the genus *Mastadenovirus* and is being characterized in detail to develop it as gene delivery vector for cattle and humans (Zakhartchouk et al., 1999; Zakhartchouk et al., 2007; Du and Tikoo, 2010). Adenoviral genes can be classified as the early, intermediate and late based on the onset of their expression before or after the viral DNA replication. Early proteins are responsible for the control of cell cycle and viral protein expression leading to viral DNA replication (Berk, 2007). The late viral proteins are transcribed from the adenoviral major late promoter (MLP) after the viral DNA replication. Seven families of late genes, named L1 to L7 based on the common poly (A) signal are present in BAdV-3 (Reddy et al., 1998).

The BAdV-3 L1 family of transcripts synthesizes the four proteins namely 52K, IIIa, penton base and pVII (Reddy et al., 1998). The L2, L3 and L4 region of BAdV-3 codes for one protein each, protein V, protein X and protein VI, respectively (Reddy et al., 1998). The L5 region of BAdV-3 codes for two proteins namely hexon and adenoviral protease (Reddy et al., 1998). BAdV-3 L6 family encode four proteins namely 100K, 33K, 22K and pVIII (Reddy et al., 1998; Kulshreshtha and Tikoo, 2008). The L7 region of BAdV-3 codes for the fiber protein (Reddy et al., 1998).

Human adenovirus 100K protein, expressed at late times post infection (Cepko and Sharp, 1982) localizes to nucleus and cytoplasm (Gambke and Deppert, 1981). It is involved in translation of cellular and late adenovirus mRNAs (Cuesta et al., 2000; Xi et al., 2004), and helps in

trimerization and nuclear localization of hexon protein (Hong et al., 2005). Not much is known about BAdV-3 100K. Recently, we demonstrated that the 100K protein can interact with the 33K protein in BAdV-3 infected MDBK cells (Kulshreshtha and Tikoo, 2008). In this report, we describe the molecular characterization of the BAdV-3 100K protein and identified the necessary region for interacting with 33K protein.

3.2 Materials and methods

3.2.1 Cell lines and viruses

Madin-Darby bovine kidney (MDBK) cells were grown in the minimum essential medium (MEM) (HyClone® Laboratories, Inc.) with 10 % fetal bovine serum (FBS) (PAA Laboratories Inc.), 10 mM HEPES buffer (Life Technologies), 0.1 mM non-essential amino acids (NEAA) (Life Technologies), and 50μg/ml gentamicin (HyClone® Laboratories, Inc.). 293T (a human embryonic kidney) cells and Vero cells were grown in the Dulbecco's modified Eagle medium (DMEM) (HyClone® Laboratories, Inc.) with 10 % FBS, 10 mM HEPES buffer, 0.1 mM NEAA and 50 μg/ml gentamicin. The wild-type BAdV-3 (WBR-1 strain) was propagated as described (Reddy et al., 1998).

3.2.2 Antibody production

The anti-100K antibodies were generated using peptides synthesized on the Pioneer Peptide Synthesis System (Perkin Elmer). Two peptides corresponding to the N-terminus (amino acids 102-126) and C-terminus (amino acids 776-790) of BAdV-3 100K protein were synthesized and conjugated to keyhole limpet hemocyanin (KLH). Two rabbits were first injected with the individual peptide (500 μg/ rabbit) with Freund's complete adjuvant at day 1 by intradermal route. Peptides with the Freund's incomplete antigen were injected on 14^{th}, 28^{th} and 42^{nd} day by

subcutaneous route to the rabbits. The serum was collected on 63rd day and presence of anti-100K antibodies was verified by immunoprecipitation analysis.

3.2.3 Analysis of 100K amino acid sequence

The amino acid sequence of 100K protein was analyzed using COILS prediction program (Lupas et al., 1991). Five coiled-coil regions were identified in the 100K protein between amino acids 121-163, 234-280, 338-370, 463-497 and 680-706.

Online program NUCDISC PSORT II (Nakai and Horton, 1999) predicted the subcellular localization signals of BAdV-3 100K protein. One bipartite (amino acids 794-810) nuclear localization signal was predicted by NUCDISC PSORT II from 100K amino acid sequence. The online software, eukaryotic linear motifs (ELM), (Dinkel et al., 2012) was used for the prediction of the functional sites of interacting amino acid sequence. The homology of amino acid sequences was generated with the Clustal W program (Larkin et al., 2007).

3.2.4 Plasmid construction

The plasmids p33K.DR, pGBKT7.33K, pGADT7.33K and pGEX-33K (spliced 33K gene of BAdV-3) (Kulshreshtha and Tikoo, 2008) were a kind gift from Dr. Vikas Kulshrestha. Bimolecular fluorescence complementation (BiFC) plasmids pGN and pGC were gift from Dr. Abraham Loyter (The Hebrew University of Jerusalem, Israel) (Levin et al., 2009). Plasmids were constructed using standard molecular techniques (Sambrook and Russell, 2000) and confirmed by DNA sequencing.

(a) *pcDNA.b100K*. A 2550 bp DNA fragment containing 100K ORF was amplified by PCR using the primers b100K·F and b100K·R (Table 3.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1 (+) (Invitrogen) creating plasmid pcDNA.b100K.

(b) *pb100K.EY*. A 2550 bp DNA fragment containing100K ORF was amplified by PCR using primers b100EY·F and b100EY·R (Table 3.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR product was digested with *Hind*III-*Age*I and ligated to *Hind*III-*Age*I digested plasmid pEYFP-N1 (Clontech) creating plasmid pb100K.EY.

(c) *pHA.b100K*. Two complementary oligonucleotides (HA·Link1 and HA·Link2) (Table 3.1) designed to encode HA epitope with four base overhangs compatible with *Hind*III cut site were annealed to create the linker. HA epitope coding linker was ligated to *Hind*III digested plasmid pcDNA.b100K creating plasmid pHA.b100K.

(d) *pGBKT7.b100K*. A 2550 bp DNA fragment containing 100K ORF was amplified by PCR using the primers GBKb100K·F and GBKb100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The amplified product was digested with *Nde*I-*Eco*RI and ligated to *Nde*I-*Eco*RI digested plasmid pGBKT7 (Clontech) creating plasmid pGBKT7.b100K.

(e) *pGADT7.b100K*. A 2550 bp DNA fragment containing 100K ORF was amplified by PCR using the primers GBKb100K·F and GBKb100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The amplified product was digested with *Nde*I-*Eco*RI and ligated to *Nde*I-*Eco*RI digested plasmid pGADT7 (Clontech) creating plasmid pGADT7.b100K.

(f) *pcDNA.b100K(1-624)*. A 1494 bp DNA fragment containing the N-terminus 624 amino acids coding region of 100K gene was PCR amplified using primers b100K·F and b100K(1-624)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The amplified PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(1-624).

Table 3.1 List of oligonucleotides

Oligo name	Sequence (5′-3′)[a]
b100K·F	AGACCCAAGCTTATGGCAGAGAAAGGCAGTGAAAATC
b100K·R	GGGCCCTCTAGACTACTCTTCTTGCCCTGGCC
HA·Link1	AGCTGAATTCGCCGCCATGGATTACCCATACGACGTACCAGATTACGCTA
HA·Link2	AGCTTAGCGTAATCTGGTACGTCGTATGGGTAATCCATGGCGGCGAATTC
GBKb100K·F	GAATTCCATATGATGGCAGAGAAAGGCAGTG
GBKb100K·R	ACTATGGAATTCCTACTCTTCTTGCCCTGGCCCTTCTT
b100EY·F	GAGCTCAAGCTTATGGCAGAGAAAGGCAGTGAAAATC
b100EY·R	GTGGCGACCGGTCTCTTCTTGCCCTGGCCCTTC
b100K(1-624)·R	TATACCTCTAGACTACTGAATCTCGAAGGTACCGA
b100K(1-637)·R	TATACCTCTAGACTAGGGCGGTTTGGTCGGCTGTC
b100K(624-637d)·F	ACCATAGCTCAAGACCTAGGTCTCATGGAATGCTACTGTCGC
b100K(624-637d)·R	GCGACAGTAGCATTCCATGAGACCTAGGTCTTGAGCTATGGT
CAT.SbfI.F	CCTGCAGGTACCCAATACTCTTCATC
CAT.SbfI.R	CCTGCAGGTAAGCTGCAATAAACAAG
b100K(121-163d)·F	CGCGACGAGGCCTTCGTACGTGCCCAAGGTACCACCCAAGAAG
b100K(121-163d)·R	CTTCTTGGGTGGTACCTTGGGCACGTACGAAGGCCTCGTCGCG
b100K(234-280d)·F	GACTCCTTACCGTGCTTCCCCACACTGTCTTTGACACATTTCG
b100K(234-280d)·R	CGAAATGTGTCAAAGACAGTGTGGGGAAGCACGGTAAGGAGTC
b100K(338-370d)·F	GACCCTGAACTTGCTCGCTGGGAGTGCCTGCACAAGTTTTTT
b100K(338-370d)·R	AAAAAACTTGTGCAGGCACTCCCAGCGAGCAAGTTCAGGGTC
b100K(463-497d)·F	CAGACTGCCATGGGCATTTGGCTAGCTGACATAGTGTTCCCCC
b100K(463-497d)·R	GGGGGAACACTATGTCAGCTAGCCAAATGCCCATGGCAGTCTG
b100K(680-706d)·F	AAAGCCGAAAGTGACCCCCAGCGCTCACGGAGTGTATCTGGACC
b100K(680-706d)·R	GGTCCAGATACACTCCGTGAGCGCTGGGGGTCACTTTCGGCTTT
GN·b100K·F	TGTCGCCCCGGGATGGCAGAGAAAGGCAGTGAAAATCAGC
GN·b100K·R	AAAGCTGAATTCCTACTCTTCTTGCCCTGGCCCTTC
GC·b33K·F	GTCGACCCCGGGATGAAACCCCGCAGCATGTCGGCAGC
GC·b33K·R	AAAGGCCGCGGCCGCTTAGGCGGGTCCGGATTCGTCC

Table 3.1 cont

Oligo name	Sequence (5′-3′)[a]
b100K·742·F1	GCTTGTGTCATCACTGAAGAAAAAGTTTTAG
b100K·742·R2	AGGGCGAGGGCGCGCCGCGCTGGCACTCCGGCGGCTC
b100K·742·R1	GTCCAGAGACCTGCAGTCAGCCTTAAGATCTCGAAGGTACCGATAAC
b100K·742·F2	GTTATCGGTACCTTCGAGATCTTAAGGCTGACTGCAGGTCTCTGGAC

[a] Restriction enzyme sites are underlined.

(g) *pcDNA.b100K(1-637).* A 1938 bp DNA fragment containing the N-terminus 637 amino acids of 100K gene was amplified by PCR using the primers b100K·F and b100K(1-637)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The amplified PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(1-637).

(h) *pcDNA.b100K(624-637d).* A 822 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(624-637d)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 1530 bp fragment of 100K gene was amplified by PCR using the primers b100K(624-637d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(624-637d).

(i) *pcDNA.b100K(121-163d)c1*. A 393 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(121-163d)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 2098 bp fragment of 100K gene was amplified by PCR using the primers b100K(121-163d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA b100K(121-163d)c1.

(j) *pcDNA.b100K(234-280d)c2*. A 733 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(234-280d)·R (Table 3.1), and plasmid pcDNA.b100K DNA

as a template. Secondly, a 1746 bp fragment of 100K gene was amplified by PCR using the primers b100K(234-280d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(234-280d)c2.

(k) *pcDNA.b100K(338-370d)c3.* A 1044 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(338-370d)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 1476 bp fragment of 100K gene was amplified by PCR using the primers b100K(338-370d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(338-370d)c3.

(l) *pcDNA.b100K(463-497d)c4*. A 1420 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(463-497d)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 1095 bp fragment of 100K gene was amplified by PCR using the primers b100K(463-497d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(463-497d)c4.

(m)*pcDNA.b100K(680-706d)c5*. A 2068 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(680-706d)·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 472 bp fragment of 100K gene was amplified by PCR using the primers b100K(680-706d)·F and b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(680-706d)c5.

(n) *pGN.b100K*. A 2550 bp DNA fragment containing 100K ORF was amplified by PCR using the primers GN·b100K·F and GN·b100K·R (Table 3.1), and plasmid pcDNA.b100K DNA as a template. The PCR amplified product was digested with *Xma*I-*Eco*RI and ligated to *Xma*I-*Eco*RI digested plasmid pGN (Levin et al., 2009) creating plasmid pGN.b100K.

(o) *pGC.33K*. A 837 bp DNA fragment containing 33K ORF was amplified by PCR using the primers GC·33K·F and GC·33K·R (Table 3.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR amplified product was digested with *Xma*I-*Not*I and ligated to *Xma*I-*Not*I digested plasmid pGC (Levin et al., 2009) creating plasmid pGC.33K.

3.2.5 Construction of full length BAdV-3 genomic clone expressing mutant 100K protein in a plasmid

(a) *pUC18.BAV3.100K*. A 9962 bp *Eco*RV-*Nde*I fragment of plasmid pUC304a+ (Du and Tikoo, 2010) was digested with *Sal*I. A 6477 bp *Sal*I-*Eco*RV fragment was isolated and ligated to *Sal*I-*Sma*I digested plasmid pUC18 creating plasmid pUC18.BAV3.100K.

(b) *pUC18.BAV3.CAT.SbfI*. A 894 bp DNA fragment containing chloramphenicol acetyltransferase (CAT) cassette was amplified by PCR using the primers CAT.SbfI.F and

CAT.SbfI.R (Table 3.1), and plasmid pBAC-lacZ (Addgene plasmid) DNA as a template. The PCR product was digested with *Nhe*I-*Afe*I (end filled-in) and ligated to *Nhe*I-*Afe*I (end filled-in) digested plasmid pUC18.BAV3.100K creating plasmid pUC18.BAV3.CAT.SbfI.

(c) *pUC304a.CAT.SbfIΔ100K*. A 4491 bp *Bam*HI DNA fragment of plasmid pUC18.BAV3.CAT.SbfI was isolated and recombined with *Mfe*I digested plasmid pUC304a (Du and Tikoo, 2010) in BJ5183 *E. coli* cells creating plasmid pUC304a.CAT.SbfIΔ100K.

(d) *pUC304aΔ100K*. Plasmid pUC304a.CAT.SbfIΔ100K DNA was digested with *Sbf*I and self-ligated to create plasmid pUC304aΔ100K.

(e) *pUC18.100K.624-637d*. A 219 bp DNA fragment of 100K gene was amplified by PCR using primers b100K·742·F1 and b100K·742·R1 (Table 3.1), and plasmid pUC18.BAV3.100K DNA as a template. Secondly, a 631 bp DNA fragment of 100K gene was amplified by PCR using primers b100K·742·F2 and b100K·742·R2 (Table 3.1), and plasmid pUC18.BAV3.100K DNA as a template. The third PCR was performed using the primers b100K·742·F1 and b100K·742·R2 (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Asc*I and ligated to *Afe*I-*Asc*I digested plasmid pUC18.BAV3.100K creating plasmid pUC18.100K.624-637d.

(f) *pUC304a.624-637d*. A 5122 bp *Sal*I-*Cla*I DNA fragment of plasmid pUC18.100K.624-637d was recombined with *Sbf*I digested plasmid pUC304aΔ100K in BJ5183 *E. coli* creating plasmid pUC304a.624-637d.

(g) *pUC304aΔ100K Rev1*. A 6163 bp *Sal*I-*Bgl*II DNA fragment of plasmid pUC18.BAV3.100K was recombined with *Mfe*I digested plasmid pUC304aΔ100K in BJ5183 *E. coli* creating plasmid pUC304aΔ100K Rev1.

(h) *pUC304a.624-637d Rev2*. A 6163 bp *Sal*I-*Bgl*II DNA fragment of plasmid pUC18.BAV3.100K was recombined with *Mfe*I digested plasmid pUC304a.624-637d in BJ5183 *E. coli* creating plasmid pUC304a.624-637d Rev2.

3.2.6 Transfection

The 293T or Vero cells (70-80% confluent) in the 24-well plate or chambered glass slides were washed with OptiMEM® (Life Technologies). After 3h of incubation, the cells were transfected with respective plasmid DNA (2 μg/well; 2 μg/chamber) using Lipofectamine^{TM} 2000 (Life Technologies) as per manufacturer's instructions.

3.2.7 Western blot analysis

MDBK cells were infected with virus at an MOI of 5. At indicated times post infection, the cells were washed once in PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and lysed for 30 min in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8)) with protease inhibitors (aprotinine, leupeptin, pepstatin A 1 μg/mL each). Proteins from the lysates of the cells were separated by 15% SDS PAGE, blotted on nitrocellulose membrane (Bio-Rad, 162-0115) and probed with primary antibody (anti-100K or anti-actin) followed by alkaline phosphatase conjugated secondary antibodies as described (Kulshreshtha et al., 2004). Finally, BCIP®/NBT solution (Sigma, B6404) was used as substrate to visualize the protein bands of interest on the nitrocellulose membrane.

3.2.8 Yeast two-hybrid system

Initially, Yeast Matchmaker GAL4 Two-Hybrid System (Clontech) was used to confirm interaction between 100K and 33K protein of BAdV-3. Assay was performed according to the

supplier's instruction manual (Clontech). Briefly, equal amount (0.1µg) of bait and prey plasmid DNA were co-transformed into AH109 strain of *Saccharomyces cerevisiae* cells using salmon sperm DNA as carrier employing lithium acetated based method. Three selective dropout agar plates (-Leu/-Trp, -Leu/-Trp/-His, -Leu/-Trp/-His/-Ade/X-alpha-Gal) were plated with transformants for growth and color development. Leaky expression of His3 was prevented by adding competitive inhibitor of His3, 3-amino-1,2,4-triazole (Sigma) at 5 mM concentration to -Leu/-Trp/-His and -Leu/-Trp/-His/-Ade/X-alpha-Gal dropout plates. Plates were incubated at 30°C in the humidified incubator for the period of 7 days. Plasmid pair pGBKT7.p53 and pGADT7.T served as positive control and plasmid pair pGBKT7.Lam and pGADT7.T was used as negative control as per manufacturer's instructions.

3.2.9 GST pull down assay

E. coli BL21 (DE3) pLysS (Promega) cells were transformed with plasmid pGEX-33K (Kulshreshtha and Tikoo, 2008) or pGEX-5X-1 DNA. The GST or GST-33K protein expression was induced by adding 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) to the exponentially growing bacterial culture on shaking incubator for 3 h at 37 °C. The proteins were purified, immobilized on Glutathione-Sepharose beads (Pharmacia), as described (Zhou et al., 2001) Protein concentration of the purified GST fusion protein was estimated by Bradford assay (Bradford. 1976). Identities of purified proteins were determined by Western blot analysis.

TNT T7 Coupled Reticulocyte Lysate System (Promega) was used for *in vitro* transcription and translation (IVTT) of individual plasmid DNA (1 µg) in the presence of 30 µCi of [^{35}S] methionine as per manufacturer's protocol in 50µl volume. About 15 µg of purified GST or GST-33K protein and 20 µl of IVTT product were added to the 40 µl of glutathione bead slurry (50%) in separate tubes and incubated on nutator for overnight at 4°C in total volume of 500 µl GST

binding buffer. Unbound IVTT product was removed by four washes (five minutes each) with 500 µl GST binding buffer on nutator at 4 °C. Finally, the beads were resuspended in 30 µl of 5X SDS sample buffer. Interacting radiolabelled protein was separated by 8% SDS-PAGE analysis, exposed overnight to phosphorimaging screen (Kodak) and visualized on Molecular Imager FX (Bio-Rad) using Quantity One software (Bio-Rad).

3.2.10 Bimolecular fluorescence complementation assay

The assay was performed as described previously with minor modifications (Levin et al., 2009). Plasmid DNA encoding the fusion proteins of N and C-terminus of green fluorescent protein (GFP) were cotransformed into yeast cells. Yeast transformation mixture were plated on the uracil and histidine deficient agar plates in humidified incubator at 30 °C for 5 days. A few colonies from each plate were resuspended into the 1 ml of PBS and washed 5 times. Yeast cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, and permeabilized by 0.5% Triton X-100 in PBS for 15 minutes. Cells were transferred onto the glass slide and mounted with VECTASHIELD® mounting medium (Vector Laboratories, H-1200). Cells were observed under the Zeiss LSM 5 confocal microscope (Carl Zeiss) for the identification of interaction.

3.2.11 Immunofluorescence microscopy

MDBK infected with wild-type BAdV-3 at an MOI of 1 or Vero cells transfected with plasmid DNA (2 µg/coverslip). Forty eight hours post transfection/infection, the cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature, and permeabilized by 0.5% Triton X-100 in PBS for 15 minutes. The cells were stained with anti-100K sera and stained with Cy2 AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories). The cells were washed in PBS three times each for 10 minutes and later counter stained with 4',6-diamidino-2-phenylindole (DAPI) followed by three more washes. Finally, the coverslips were mounted with

Citifluor AF4 mounting media. Images were captured using different laser lines under laser scanning confocal microscope (Zeiss LSM410).

3.3 Results

3.3.1 Expression of 100K protein

Protein specific antisera designated N100 (against N-terminus peptide) and C100 (against C-terminus peptide), generated separately in rabbits was used to characterize 100K protein in gene transfected or BAdV-3 infected cells by Western blot analysis (Fig. 3.1). Anti-HA monoclonal antibody recognized a doublet of 130 kDa in plasmid pHA.b100K transfected cells (panel A). No such protein could be detected in plasmid pc.HA (pcDNA containing HA tag) transfected cells. Anti-100K (N100+C100) sera detected a protein of 130 kDa in BAdV-3 infected cells from 24-48 hrs post infection (panel B). Moreover, additional bands of 100 kDa, 95 kDa and 15 kDa were also detected at 36-48 hrs post infection which may be due to post-translational modification or protein cleavage. No such proteins could be detected at 0-12 hrs post infection. Earlier, 33K protein was detected at the 24, 36 and 48 hrs post infection as a protein of 42 kDa in infected cell lysates (Kulshreshtha et al., 2004). Moreover, additional bands of 33 and 38 kDa were also detected at 36-48 hrs post infection (Kulshreshtha et al., 2004).

3.3.2 Sub-cellular distribution of 100K

To determine subcellular localization of proteins in infected cells, MDBK cells on the glass coverslips were infected with BAdV-3 at an MOI of 5. At 48 hrs post infection, the cells were examined by indirect immunofluorescence using protein specific sera. As seen in figure 3.2A, anti-100K (N100+C100) sera detected protein in the nucleus and cytoplasm of infected cells. Similarly,

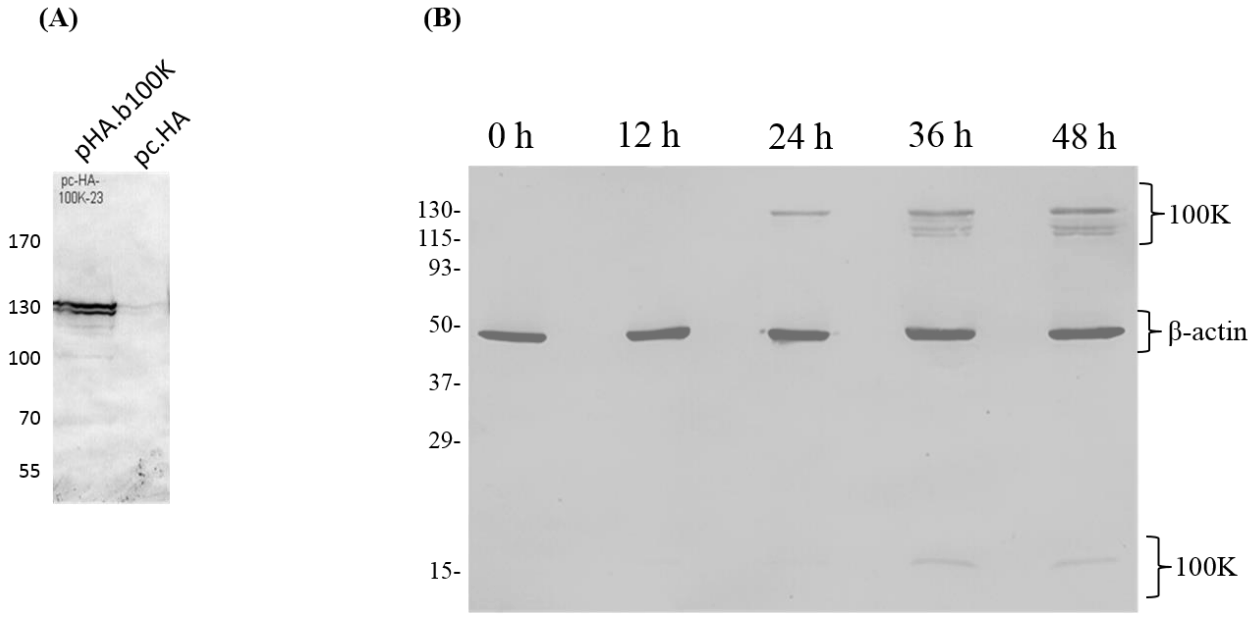

Figure 3.1 Expression of 100K protein. Proteins from the lysates of 293T cells transfected with indicated plasmid DNAs **(panel A)** collected after 48 hrs post transfection or BAdV-3 infected MDBK, collected at indicated times post infection **(panel B)** were separated by 12 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-HA monoclonal antibody **(panel A)** or anti-100K sera (N100+C100) + anti-β actin monoclonal antibody **(panel B)**. Molecular weight markers are shown on the left of the panel.

anti-33K (Kulshreshtha and Tikoo, 2008) detected protein predominantly localized in the nucleus of infected cells.

To determine the subcellular localization of proteins in transfected cells, Vero cells were transfected with indicated plasmid DNA. At 48 hrs post transfection, cells were examined by direct fluorescence. As seen in figure 3.2B 100K-EYFP fusion protein localized predominantly in the cytoplasm of plasmid pb100K.EY DNA transfected Vero cells. Similarly, anti-HA (MAb) detected HA tagged 100K predominantly in the cytoplasm of the plasmid pHA.b100K DNA transfected cells. However, 33K-DsRed fusion protein localized predominantly in the nucleus of the transfected cells.

3.3.3 Interaction of 100K and 33K protein of BAdV-3

To reconfirm the interaction of 100K protein with 33K protein, initially yeast two-hybrid assay was performed. The plasmid pGBKT7.100K and pGADT7.33K DNAs were cotransformed in the AH109 and grown on selective drop out media. The positive interaction was identified by the presence growth and the green color development. The reverse yeast two-hybrid assay was performed using plasmid DNA pGADT7.100K and pGBKT7.33K and the interaction was confirmed as growth and color development on selective drop out media. The plasmid positive and negative controls were used as per manufacturer's instructions. As seen in figure 3.3, 100K interacted with 33K protein.

Secondly, we tested the interaction of 100K with 33K by GST pull-down assay. Purified GST or GST-33K (Fig. 3.4, panel A) bound to glutathione Sepharose beads were individually incubated with *in vitro* translated [^{35}S] labeled 100K for 4 hrs at 4°C. The bound products were separated by 10 % SDS-PAGE and visualized by phosphorimager. As seen in figure 3.4 (panel B), *in vitro* translated labeled 100K bound to GST-33K but not to GST alone.

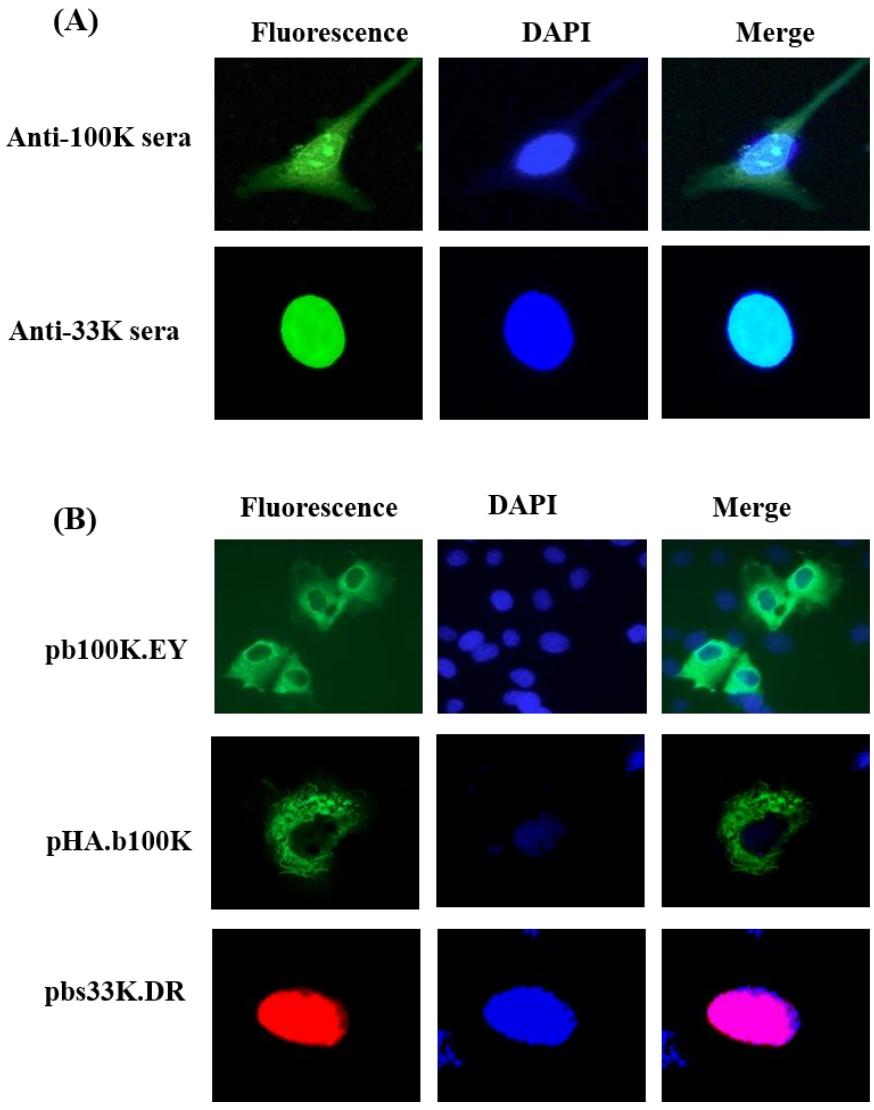

Figure 3.2 Subcellular distribution of BAdV-3 100K protein in infected and transfected cells. **(A)** BAdV-3 infected MDBK cells were processed at 48 h p.i. for confocal microscopy analysis for subcellular distribution of 100K protein (green) using anti-100K or anti-33K primary antibody and Cy2 AffiniPure Goat Anti-Rabbit IgG as secondary antibody (see Materials and Methods). Nuclear demarcation was achieved by staining with DAPI (blue). **(B)** Vero cells were transfected with plasmid DNA mentioned on the left side were processed at 48 h p.t. for confocal microscopy analysis for subcellular distribution of 100K protein (green) or 33K protein (red).

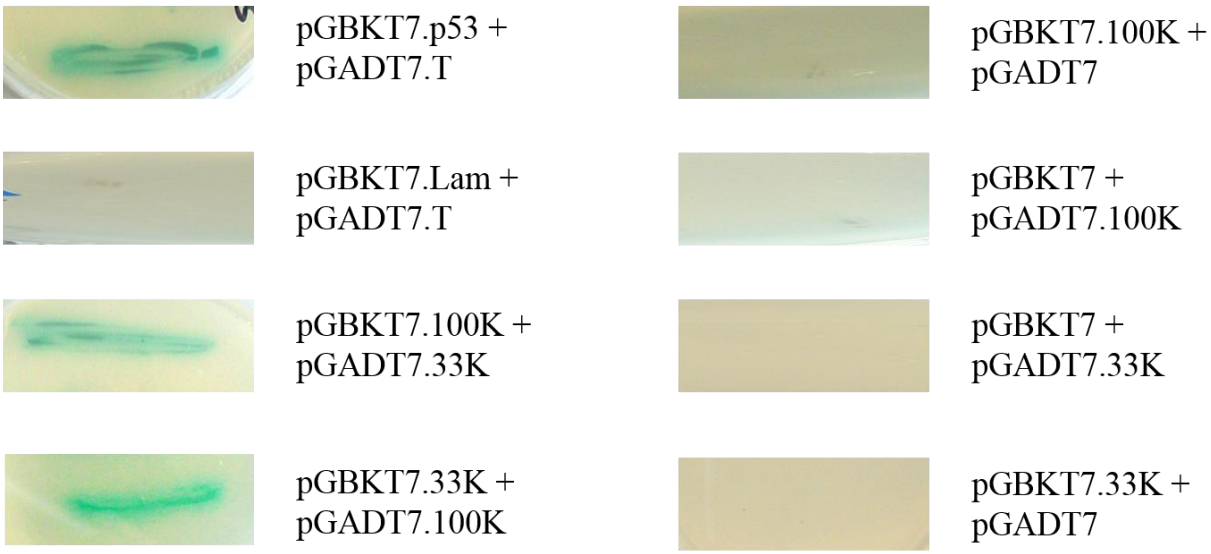

Figure 3.3 Confirmation of interaction of 100K-33K proteins using yeast two-hybrid assay. The AH109 cells were transformed with the bait and prey plasmids (mentioned on the right side of each photograph) and plated on the –leucine, –tryptophan, –histidine, –adenine plates supplemented with the X-alpha-gal. Plasmids pGBKT7.p53 and pGADT7.T were used as positive controls for the assay.

Finally, the interaction between 100K and 33K proteins was confirmed using the bimolecular fluorescence complementation assay. The 100K protein coding DNA was fused in-frame with the N-terminal half of GFP protein coding region to create the plasmid pGN.100K. The spliced 33K protein coding DNA was fused in-frame with the C-terminal half of GFP protein coding region to create the plasmid pGC.33K. The co-transformants were analyzed by confocal microscopic examination and the green fluorescence was visible indicating the interaction between the 100K and 33K proteins of BAdV-3. As seen in figure 3.5, yeast cells co-transformed with pGN.100K+pGC.33K showed green fluorescence. No such fluorescence could be observed in yeast cells co-transformed with pGN.100K + pGC.33K or pGC.33K + pGN.

3.3.4 Co-expression of 100K and 33K proteins

To determine if interaction between 100K and 33K proteins alters the subcellular distribution of 100K protein in transfected cells, Vero cells were co-transfected with plasmid (pb100K.EY and p33K.DR) DNAs using the lipofectamine 2000 reagent and analyzed by direct fluorescence after 48 hrs of transfection. As seen in figure 3.5 (panel B), co-expression of 33K-DsRed fusion protein did not alter the predominant cytoplasmic localization of 100K-EYFP fusion.

3.3.5 Identification of 100K protein domain interacting with 33K protein

To determine the domain of 100K interacting with 33K, initially we constructed plasmids containing deletions of individual coil-coil regions of 100K (Fig. 3.6, panel A) and analyzed interactions using GST-pull down assay. The deletions were analyzed by DNA sequencing and Western blot analysis using anti-100K (N100+C100) (Fig. 3.6 panel B). *In vitro* transcribed and translated (in the presence of 30 μCi of [^{35}S] methionine) mutant 100K proteins were individually incubated with GST or GST fusion proteins bound to Glutathione Sepharose beads. The bound

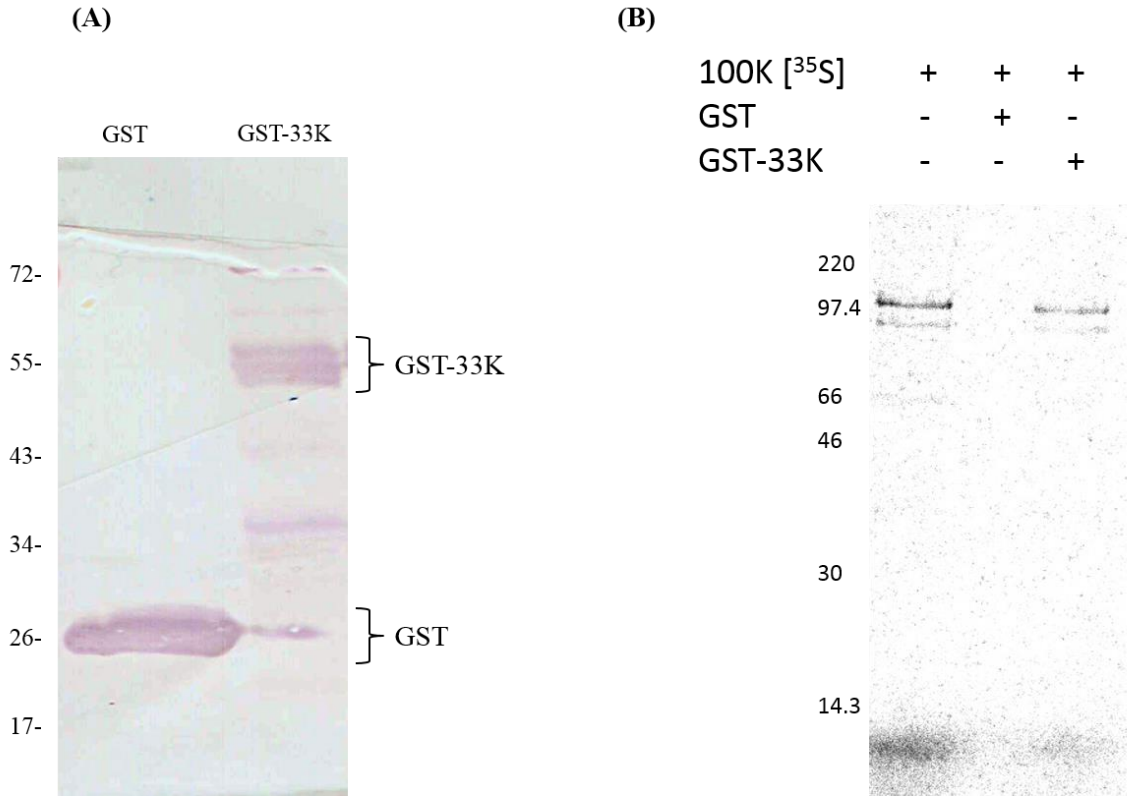

Figure 3.4 GST pull-down assay. (A) Purified proteins were separated by 10 % SDS-PAGE and analyzed by immunoblotting using anti-GST serum. The position of the molecular weight markers in kDa is shown to the left of the panel. **(B)** The *in vitro* transcribed/translated radiolabelled 100K protein was incubated with the purified GST alone or GST-33K fusion protein. The bound protein was separated by 10 % SDS PAGE and visualized by exposure to the phoshorimager screen. Input radiolabelled protein was 5% of the reaction volume. The molecular weights in kDa are indicated on the left side of the figure.

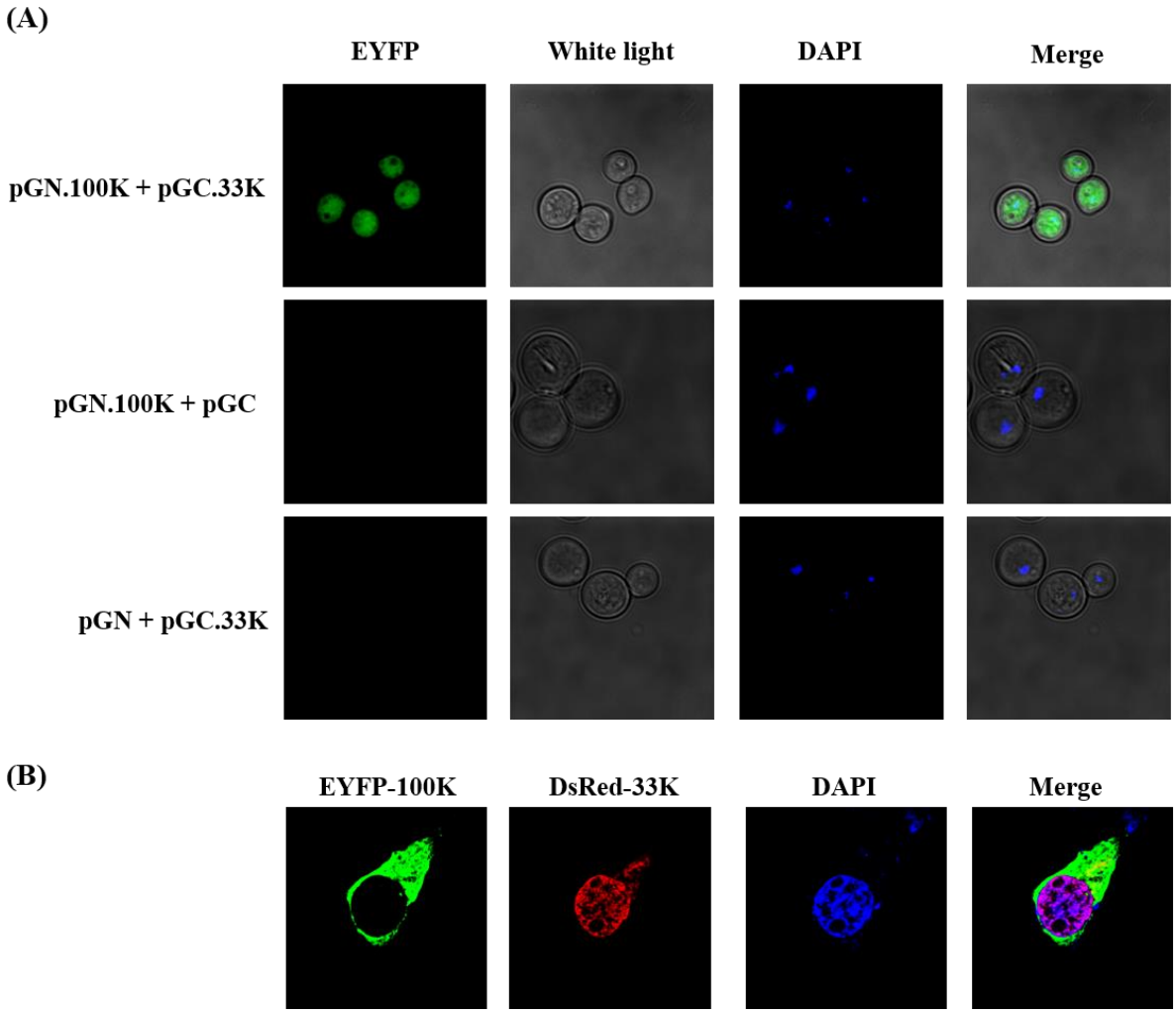

Figure 3.5 Confirmation of 100K protein interaction with 33K protein and its effect on localization. (A) Yeast cells were co-transformed with the indicated plasmid DNAs. Co-transformed cells were plated on the uracil and histidine deficient agar plates and grown at 30° C for 5 days and processed for confocal microscopy for the presence of the green fluorescence. Empty vector pGN and pGC were used as negative controls for the self-complementation. **(B)** Vero cells were co-transfected with the plasmid pb100K.EY and p33K.DR DNA and processed at 48 hrs p.t for confocal microscopy analysis.

labeled proteins were separated by 10 % SDS-PAGE and visualized by phosphorimager. As seen in figure 3.6 (panel C), all mutant 100K proteins bound to GST-33K fusion protein.

Next, we constructed two plasmids expressing truncated 100K proteins (Fig. 3.7, panel A, B) and analyzed the interaction of these proteins with GST-33K in GST-pull down assay. As seen in figure 3.7C, wild-type 100K or mutant 100K (expressing amino acids 1-637) interacts with GST-33K fusion protein. In contrast, no interaction could be detected between mutant 100K (expressing amino acids 1-624) and GST-33K fusion protein. No interaction was detected between wild-type or mutant 100K proteins and GST alone. Based on these results, a GST pull-down assay was performed with mutant 100K containing deletion between amino acid 624-637. As seen in figure 3.7 (panel C), mutant 100K containing deletion of amino acid 624-637 abolished the interaction of 100K protein with 33K.

To validate these results *in vivo,* a bimolecular fluorescence complementation assay was performed. As seen in figure 3.8, green fluorescence could be observed in yeast cells cotransformed with plasmid pGN.100K+pGC.33K DNAs or pGN.100K(1-637) + pGC.33K DNAs. No such fluorescence could be observed in yeast cells cotransformed with plasmid pGN. 100K (1-624) + pGC.33K; pGN.100(624-638)+ pGC.33K, pGN100K + pGC or pGN + pGC.33K DNAs.

3.3.6 Isolation and analysis of BAdV-3 expressing mutant 100K protein

The construction of plasmid pUC304a+ has been described (Du and Tikoo, 2010). Using recombination machinery of *E. coli* (Chartier et al., 1996), we constructed full length plasmids a) pUC304aΔ100K mutant 100K protein (deletion of amino acid 19 to 678), b) pUC304a.624-637 expressing mutant 100K (deletion of amino acids 624-637). We also constructed revertants a) pUC304aΔ100K Rev1 expressing wild-type 100K and b) *pUC304a.*624-637 Rev2 expressing wild

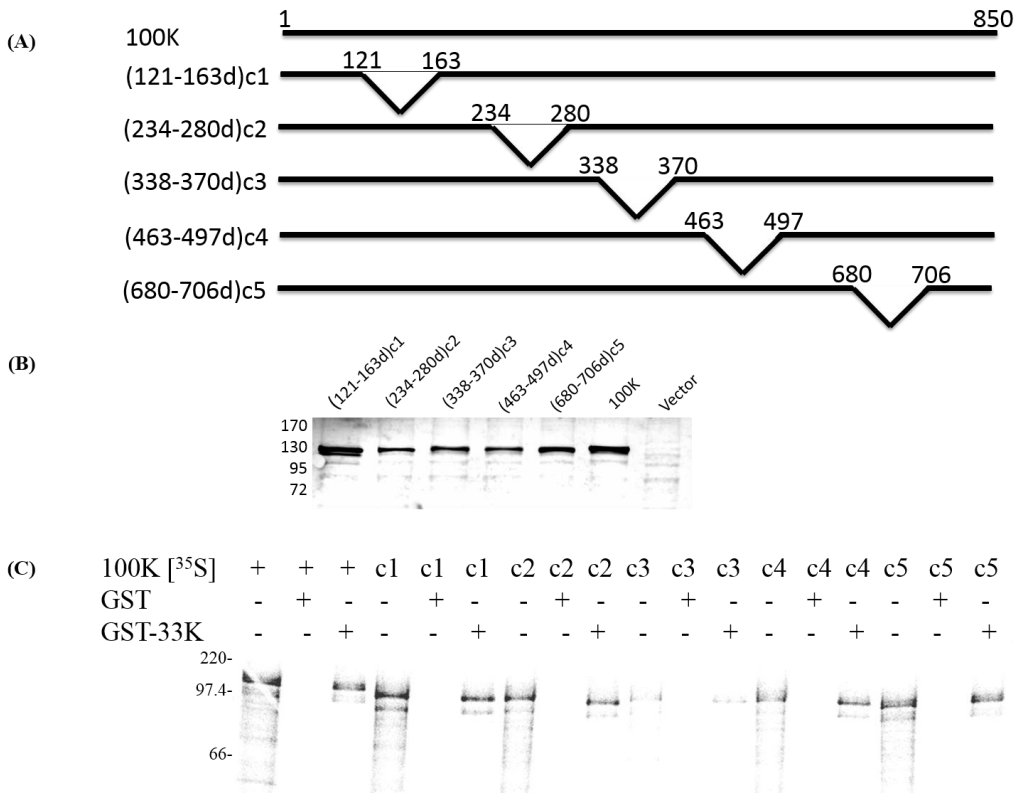

Figure 3.6 Interaction of coiled coils deletions of 100K proteins with GST-33K protein by GST pull-down assay. (A) Schematic representation of the plasmid DNA. The amino acid numbers are depicted on the top of each construct. The name of the protein is depicted on the left of the panel. **(B)** Proteins from the lysates of indicated plasmid DNA transfected 293T cells collected after 48 hrs post transfection were separated by 10 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-100K (N100+C100) sera. **(C)** The *in vitro* transcribed/translated [^{35}S] labelled indicated proteins were incubated with the purified GST alone or GST-33K proteins. The bound proteins were separated by 10 % SDS PAGE and visualized by exposure to the phoshorimager screen. Input radiolabelled protein was 5% of the reaction volume. The molecular weights are indicated on the left side of the figures as numerals in kDa; c1 [pcDNA b100K(121-163d)c1]; c2 [pcDNA.b100K(234-280d)c2]; c3 [pcDNA.b100K(338-370d)c3]; c4 [pcDNA.b100K(463-497d) c4]; c5 [pcDNA.b100K(680-706d)c5]; 100K [pcDNA.b100K].

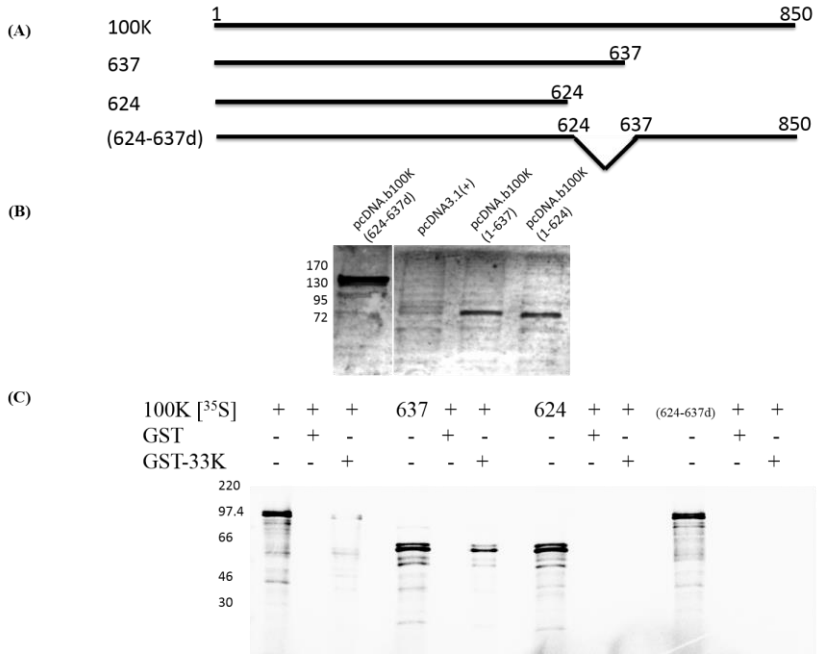

Figure 3.7 Identification of 100K protein region interacting with GST-33K protein by GST pull-down assay. (A) Schematic representation of the plasmid DNA. The amino acid numbers are depicted on the top of each construct. The name of the protein is depicted on the left of the panel. **(B)** Proteins from the lysates of indicated plasmid DNA transfected 293T cells collected after 48 hrs post transfection were separated by 10 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-100K (N100+C100) sera. **(C)** The *in vitro* transcribed/translated [^{35}S] labelled indicated proteins were incubated with the purified GST alone or GST-33K proteins. The bound proteins were separated by 10 % SDS PAGE and visualized by exposure to the phoshorimager screen. Input radiolabelled protein was 5% of the reaction volume. The molecular weights are indicated on the left side of the figures as numerals in kDa; 637 [pcDNA b100K(1-637)]; 624 [pcDNA.b100K(1-624)]; (624-637d) [pcDNA.b100K (624-637d)]; 100K [pcDNA.b100K].

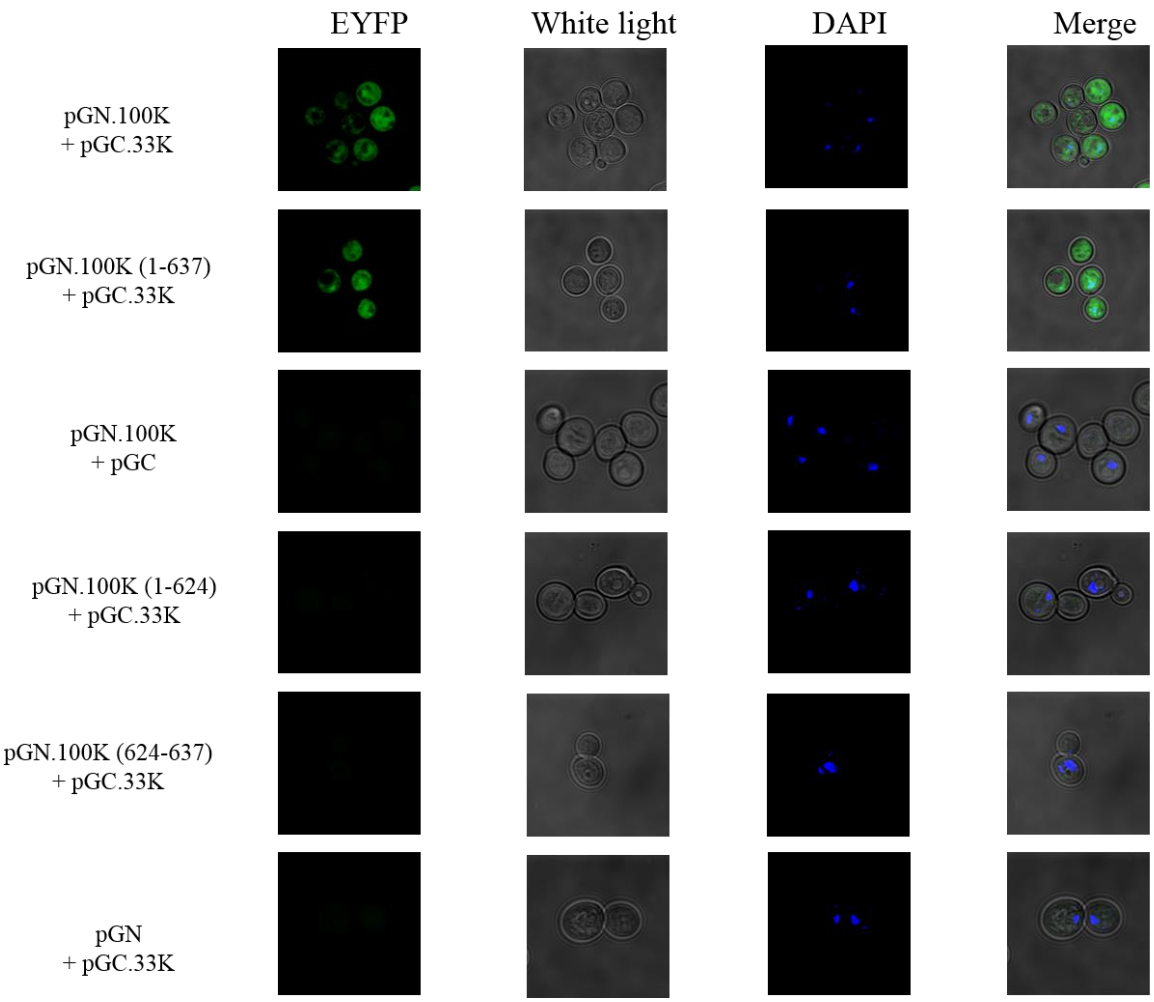

Figure 3.8 Interaction of 100K-33K proteins using bimolecular fluorescence assay. Yeast cells were co-transformed with the plasmids mentioned on left side of each panel. Co-transformed cells were plated on the uracil and histidine deficient agar plates and grown at 30° C for 5 days and processed for confocal microscopy for the presence of the green fluorescence. Empty vector pGN and pGC were used as negative controls for the self complementation.

-type 100K. The desired mutation was confirmed by restriction enzyme analysis (data not shown) and DNA sequencing of recombinant plasmid DNAs. The plasmid pUC304a+, pUC304aΔ100K Rev1 or pUC304a.624-627 Rev2 DNAs when individually transfected into VIDO DT1 (Du and Tikoo, 2010) cells produced cytopathic effects in 14 days. Restriction enzyme analysis of virion DNA and growth in MDBK cells suggested that wild-type and revertant BAdV-3 possess similar characteristics. However, repeated (7 times) transfection of VIDO DT1 cells with varying amounts of plasmid pUC304a.624-637 DNA did not produce cytopathic effects even after four weeks of transfection.

3.4 Discussion

Protein-protein interactions play an important role in host-pathogen interactions. Most viruses encode a limited number of the proteins and the interactions among those proteins may help in performing multiple functions. The 100K protein acts as chaperon for trimerization of hexon protein in HAdV-5 and HAdV-2 infected cells (Oosterom-Dragon and Ginsberg 1981; Hong et al., 2005; Cepko and Sharp, 1982) and is involved in preventing apoptosis by granzyme B (Andrade et al., 2001) Here, we report the characterization of BAdV-100K protein and demonstrate that amino acid 624-637 involved in the interaction with 33K protein may be essential for the replication of BAdV-3.

The BAdV-3 100K is predicted to encode 850 amino acids. Although a protein of 130 kDa could be detected at early (24 hrs) post infection, multiple bands could be detected at late times (36-48 hrs) post infection. It is possible that different forms of 100K protein could arise due to post translation modifications as observed in HAdV-5 infected cells (Xi et al., 2005; Koyuncu and Dobner, 2009). Alternatively, different forms of 100K could arise due to proteolytic cleavage. Although 100K protein is localized in the nucleus and cytoplasm of BAdV-3 infected cells, it is

predominantly localized in the cytoplasm of transfected cells. In contrast, localization of HAdV-5 100K appears similar in infected/transfected cells (Koyuncu and Dobner, 2009). These results suggest that nuclear localization of BAdV-3 100K may require other viral or cellular proteins.

An earlier report suggested that HAdV-2 100K interacts with hexon (Wodrich et al., 2003). Recently, we demonstrated that 33K protein interact with 100K protein in BAdV-3 infected cells (Kulshreshtha and Tikoo, 2008). However, co-expression of 100K protein with either hexon or 33K proteins did not alter the cytoplasmic location of 100K in transfected cells. This is despite the fact that BAdV-3 100K contains a potential bipartite nuclear localization signal. It is possible that nuclear localization of BAdV-3 100K requires more than one viral/cellular protein. Alternatively, it is possible that nuclear localization of BAdV-3 100K in infected cells occurs due to yet unidentified cellular factor.

Earlier, we demonstrated that amino acid 161-200 of 33K are involved in interacting with 100K of BAdV-3 (Kulshrestha and Tikoo 2008). Using different assays including bio immunofluorescence molecular complementation assay, we reconfirmed these observations and further demonstrated that a stretch of amino acids 624-637 of 100K appears to be involved in the interaction with 33K. Although, precise location and functional importance of the interacting domain in the different steps of BAdV-3 replication is not known yet, it is noteworthy to suggest that 100K-33K interaction may have a role in the event occurring in the cytoplasm of infected cells. Moreover, amino acid 1-742 containing the interacting domain appear to be retained in the cytoplasm of cell (chapter 5).

Like HAdV-2 (Xi et al., 2004), 100K protein appears to be essential for the replication of BAdV-3. The inability to isolate mutant BAdV-3 expressing 100K containing a deletion of 624-637 amino acids suggests that the 100K-33K interaction is essential for BAdV-3 replication.

Alternatively, deletion of 14 amino acid interacting region of 100K may disrupt other unknown vital function. Although analysis of 14 amino acids did show the presence of a non-canonical class I SH3 ligand specific motif (DGQPTKP) (Dinkel et al., 2012), amino acid homology search reveled 30.77% identity in similar region among 100K proteins encoded by different *Mastadenoviruses* (HAdV-2 and HAdV-5). It would be interesting to determine the role of specific amino acids by constructing point mutants that prevent interaction and determine the importance of these in different steps of BAdV-3 life cycle.

4.0 INTERACTION OF BAdV-3 100K PROTEIN WITH CELLULAR PROTEIN Tctex-1

4.1 Introduction

Adenoviruses are the large family of medium sized, non-enveloped, double stranded DNA viruses which can infect broad range of vertebrate hosts (Berk, 2007). Like other viruses, adenoviral proteins interact with the cellular proteins during the infection process of cells. Some of the adenoviral proteins and cellular proteins interactions include fiber knob domain and coxsackie and adenovirus receptor (Cupelli and Stehle, 2011; Arnberg. 2012), penton base and integrins (Wickham et al., 1993), hexon and dynein intermediate chain 2 C (Bremner et al., 2009), E1A protein and retinoblastoma (Rb) protein (Ghosh and Harter, 2003), E1B-55K protein and pro-apoptotic activator protein p53 (Levine. 1997; White, 2001), E4orf6 and p73 (Steegenga et al., 1999), 100K protein and translation initiation factor eIF4G (Cuesta et al., 2004), 100K protein and human protein arginine methyltransferase 1 (Kzhyshkowska et al., 2004) and 33K protein and presenilin-1-associated protein (PSAP) (Kulshreshtha, 2009).

Dynein and kinesin motors are responsible for cargo transport towards and away from microtubule-organizing center (MTOC) on the microtubules respectively (Dodding and Way, 2011). Cytoplasmic dynein motors are high molecular weight (~1.5 mDa) complex comprising six subunits including heavy chains, intermediate chains, light intermediate chains and light chains (Pfister et al., 2005). There are six dynein light chains (10-14 kDa), which bind the various cargos designated as DYNLL1, DYNLL2, DYNLT1, DYNLT3, DYNLRB1 and DYNLRB2. Moreover, the dynein light chains DYNLT1 and DYNLT3 can form the homodimers and heterodimers (Lo et al., 2007b). Interestingly, only homodimers of the DYNLT1 and DYNLT3 can bind with the intermediate chain for transport (Lo et al., 2007b). DYNLT1 was also reported to interact with the other cellular proteins namely Doc2 (Nagano et al., 1998), neuronal voltage gated calcium

channels (Lai et al., 2005) and C-terminus of rhodopsin protein (Tai et al., 1999). DYNLT3 has been implicated to interact with the cellular protein Bub3 and have important role in mitosis progression (Lo et al., 2007a). DYNLRB1 was reported to interact with the cellular protein reduced folate carrier (Ashokkumar et al., 2009). DYNLRB2 was found to play a key role in the TGFbeta signaling through Smad3 (Jin et al., 2009). Dynein light chains DYNLL1 and DYNLL2 interact with the myosin-Va, an actin-based neuronal motor protein (Espindola et al., 2000). These observations point to additional roles of dynein light chains in transcription regulation (Crepieux et al., 1997).

Viruses use the cellular motors for the movement through the dense cytoplasm of infected cells (Lyman and Enquist, 2009). Both DNA (herpesvirus (Lyman and Enquist, 2009), adenovirus (Bremner et al., 2009), parvovirus (Suikkanen et al., 2003), baculovirus (Ohkawa et al., 2010)) and RNA (retrovirus (Lehmann et al., 2009)) viruses have been reported to utilize microtubule network for efficient infection. Although a number of proteins encoded by both DNA (VP26, VP5, US9) (Lyman and Enquist, 2009; Kratchmarov et al., 2013) and RNA (P protein, gag protein) (Raux et al., 2000; Petit et al., 2003) viruses have been reported to interact mainly with dynein light chains, the biological significance of only few has been explored.

A few adenovirus (AdV) proteins have been shown to interact with proteins of the microtubule network (Engelke et al., 2011). Interaction of viral structural proteins including adenovirus hexon with cytoplasmic dynein mediates transport of the virus across the cytoplasm of infected cells (Bremner et al., 2009). Interaction of AdV E3 nonstructural protein 19K with components of microtubules has been suggested to retain class I major histocompatibility complex molecules when complexed with gp19 into endoplasmic reticulum thus down regulating cell surface expression of MHC-1 proteins (Dahllof et al., 1991). Another adenovirus E3 encoded non-

structural protein 14.7K has been reported to interact with dynein (Lukashok et al., 2000). Although biological significance of 14.7K-dynein interaction is not clear, the potential of its role in viral spread has been proposed. Here, we report the interaction of BAdV-3 100K protein of with the dynein light chain protein DYNLT1 using the yeast two-hybrid, coimmunoprecipitation and GST pull-down assays.

Dyneins are cytoplasmic molecular motors responsible for the transport of cargo towards the negative end of microtubule (Lo et al., 2007b). The cytoplasmic dynein light chains have been divided into three main types DYNLT, DYNLRB and DYNLL, each with two isoforms (Pfister et al., 2005). Dynein light chains are link between the cargo proteins and dynein light intermediate chains. Many viral and cellular proteins have been found to bind the dynein light chains for the transport in the dense cytoplasmic environment. The dynein light chain protein was of *Culex quinquefasciatus,* which has the amino acid sequence similarity with the DYNLT1 protein of *Bos taurus*.

4.2 Materials and methods

4.2.1 Cell lines and viruses

Madin-Darby bovine kidney (MDBK) cells were propagated in the minimum essential medium (MEM) (HyClone® Laboratories, Inc., SH30024.01) supplemented with 10 % fetal bovine serum (FBS) (PAA Laboratories Inc., A15-203). 293T (a human embryonic kidney) cells and Vero cells were cultured in the Dulbecco's modified Eagle medium (DMEM) (HyClone® Laboratories, Inc., SH30022.01) supplemented with 10 % FBS. The wild-type BAdV-3 (WBR-1 strain) was propagated as described earlier (Reddy et al., 1998).

4.2.2 Plasmids

The construction of plasmid pcDNA.b100K, pGBKT7.b100K, pGADT7.b100K, pcDNA b100K(121-163d)c1, pcDNA.b100K(234-280d)c2, pcDNA.b100K(338-370d)c3, and pcDNA.b100K(463-497d)c4 has been described (section 3.2.4). Plasmids pGEX-DYNLT1, pGEX-DYNLT3 and pLexA-LC8 expressing the DYNLT1, DYNLT3 and DYNLL1 were a generous gift from Dr. Russells Diefenbach (Westmead Millennium Institute, Australia) (Douglas et al., 2004). All the cloning reactions were performed by the standard molecular techniques.

(a) *pGBKT7.DYNLT1*. A 339 bp DNA fragment containing DYNLT1 ORF was amplified by PCR using primers GBKDYNLT1·F and GBKDYNLT1·R (Table 4.1), and plasmid pGEX-DYNLT1 (Douglas et al., 2004) DNA as template. The PCR product was digested by *EcoRI-NotI* and ligated to *Eco*RI-*Not*I digested plasmid pGBKT7 (Clontech, 630489) downstream of DNA binding domain of Gal4 gene creating plasmid pGBKT7.DYNLT1.

(b) *pGADT7.DYNLT1*. A 339 bp DNA fragment containing DYNLT1 ORF was amplified by PCR using primers GADDYNLT1·F and GADDYNLT1·R (Table 4.1), and plasmid pGEX-DYNLT1 (Douglas et al., 2004) DNA as template. The PCR product was digested by *EcoRI-XhoI* and ligated to *Eco*RI-*Xho*I digested plasmid pGADT7 (Clontech, 630489) downstream of DNA activation domain of Gal4 gene creating plasmid pGADT7.DYNLT1.

(c) *pGBKT7.DYNLT3*. A 348 bp DNA fragment containing DYNLT3 ORF was amplified by PCR using primers GBKDYNLT3 F and GBKDYNLT3 R (Table 4.1), and plasmid pGEX-DYNLT3 (Douglas et al., 2004) DNA as template. The PCR product was digested by *Eco*RI-*Not*I and ligated to *Eco*RI-*Not*I digested plasmid pGBKT7 (Clontech, 630489) downstream of DNA binding domain of Gal4 gene creating plasmid pGBKT7.DYNLT3.

(d) *pGBKT7.b100K*. Construction of plasmid pGBKT7.b100K is previously described (section 3.2.4).

(e) *pGADT7.b100K*. Construction of plasmid pGADT7.b100K is previously described (section 3.2.4).

(f) *pGBKT7.DYNLL1*. A 267 bp DNA fragment containing DYNLL1 ORF was amplified by PCR using primers GBKDYNLL1 F and GBKDYNLL1 R (Table 4.1), and plasmid pLexA-LC8 (Douglas et al., 2004) DNA as template. The PCR product was digested by *Eco*RI-*Not*I and ligated to *Eco*RI-*Not*I digested plasmid pGBKT7 (Clontech, 630489) downstream of DNA binding domain of Gal4 gene creating plasmid pGBKT7.DYNLL1.

(g) *pb100K.EY*. Construction of the 100K-EYFP gene fusion expressing plasmid is previously described (section 3.2.4).

(h) *pDR.DYNLT1*. A 339 bp DNA fragment containing DYNLT1 ORF was amplified by PCR using primers DRDYNLT1·F and DRDYNLT1·R (Table 4.1), and plasmid pGADT7.DYNLT1 as template DNA. The PCR product was digested by *BamH*I-*Xba*I and ligated to *BamHI-XbaI* digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.DYNLT1.

(i) *pcmyc.DYNLT1*. A 339 bp DNA fragment containing DYNLT1 ORF was amplified by PCR using primers cmycDYNLT1·F and cmycDYNLT1·R (Table 4.1), and plasmid pGADT7.DYNLT1 as template DNA. The PCR product was digested by *EcoR*I-*Xho*I and ligated to *EcoR*I-*Xho*I digested plasmid pc.cmyc creating plasmid pcmyc.DYNLT1.

(j) *pcDNA.b100K(1-498)*. A 1494 bp DNA fragment encoding the N-terminus 498 amino acids of 100K gene was PCR amplified using the primers b100K·F and b100K(1-498) R (Table 4.1), and plasmid pcDNA.b100K (section 3.2.4) DNA as a template. The amplified PCR product

was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(1-498).

(k) *pcDNA.b100K(1-588).* A 1764 bp DNA fragment encoding the N-terminus 588 amino acids of 100K gene was PCR amplified using the primers b100K·F and b100K(1-588) R (Table 4.1), and plasmid pcDNA.b100K (section 3.2.4) DNA as a template. The amplified PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(1-588).

(l) *pcDNA.b100K(499-587d)*. A 1530 bp fragment of 100K gene was amplified by PCR using the primers b100K·F and b100K(499-587d)·R (Table 4.1), and plasmid pcDNA.b100K DNA as a template. Secondly, a 819 bp fragment of 100K gene was amplified by PCR using the primers b100K(499-587d)·F and b100K·R (Table 4.1), and plasmid pcDNA.b100K DNA as a template. The third PCR was performed using the primers b100K·F and b100K·R (Table 3.1) and both previously amplified annealed PCR products as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) creating plasmid pcDNA.b100K(499-587d).

(m)*pGN.100K*. Construction of the plasmid pGN.100K is previously described (section 3.2.4).

(n) *pGN.100K(499-587d)*. A 2280 bp DNA fragment containing 100K (499-587 amino acid deletion) ORF was amplified by PCR using the primers GN·100K·F and GN·100K·R (Table 4.1), and plasmid pcDNA.b100K DNA as a template. The PCR amplified product was digested with *Xma*I-*Eco*RI and ligated to *Xma*I-*Eco*RI digested plasmid pGN (Levin et al., 2009) creating plasmid pGN.100K(499-587d).

(o) *pGC.DYNLT1*. A 339 bp DNA fragment containing DYNLT1 ORF was amplified by PCR using primers GC DYNLT1·F and GC DYNLT1·R (Table 4.1), and plasmid pGADT7.

Table 4.1 List of oligonucleotides

Oligo name	Sequence (5′-3′)[a]
GBKDYNLT1·F	GAGGCCGAATTCATGGAAGACTACCAGGCTGC
GBKDYNLT1·R	AGTTATGCGGCCGCTGCAGGTCGAGTCAAATAGAGAGTCCGAAGG
GADDYNLT1·F	GCCAGTGAATTCATGGAAGACTACCAGGCTGC
GADDYNLT1·R	CTGCAGCTCGAGTCAAATAGAGAGTCCGAAGG
GBKDYNLT3·F	GAGGCCGAATTCATGGAGGAGTACCATCGCCA
GBKDYNLT3·R	AGTTATGCGGCCGCTGCAGGTCGAGTTAAAGAACAATAGCAATGG
GBKDYNLL1·F	GAGGCCGAATTCATGTGCGACCGAAAGGCCGTG
GBKDYNLL1·R	AGTTATGCGGCCGCTTAACCAGATTTGAACAGAAG
DRDYNLT1·F	GGCCCGGGATCCCCGGGCGAATTCATGGAAGACTACCAGGCTGC
DRDYNLT1·R	CAGTTATCTAGATGCATGCTCGAGTCAAATAGAGAGTCCGAAGG
cmycDYNLT1·F	CCGGGCGAATTCATGGAAGACTACCAGGCTGC
cmycDYNLT1·R	TGCATGCTCGAGTCAAATAGAGAGTCCGAAGG
b100K·F	AGACCCAAGCTTATGGCAGAGAAAGGCAGTGAAAATC
b100K(1-498)·R	TATACCTCTAGACTATAGGTCTTGAGCTATGGTG
b100K(1-588)·R	TATACCTCTAGACTAGATGTCGGAGTGAAACATGAG
b100K(499-587d)·F	ACCATAGCTCAAGACCTAGGTCTCATGGAATGCTACTGTCGC
b100K(499-587d)·R	GCGACAGTAGCATTCCATGAGACCTAGGTCTTGAGCTATGGT
b100EY·R	GTGGCGACCGGTCTCTTCTTGCCCTGGCCCTTC
GN·100K·F	TGTCGCCCCGGGATGGCAGAGAAAGGCAGTGAAAATCAGC
GN·100K·R	AAAGCTGAATTCCTACTCTTCTTGCCCTGGCCCTTC
GC DYNLT1·F	GTCGACCCCGGGATGGAAGACTACCAGGCTGCGGAGG
GC DYNLT1·R	AAAGGCCGCGGCCGCTCAAATAGAGAGTCCGAAGGCAC

[a] Restriction enzyme sites are underlined.

DYNLT1 as template DNA. DNA as a template. The PCR amplified product was digested with *Xma*I-*Not*I and ligated to *Xma*I-*Not*I digested plasmid pGC (Levin et al., 2009) creating plasmid pGC.DYNLT1.

4.2.3 Transfections

Vero or 293T cells at the 70% confluency were transfected with plasmid DNA (2 μg/2 x 10^5 cells) using the Lipofectamine™ 2000 (Life Technologies, 11668-019) transfection reagent as per the manufacturer's protocol. At 48 h post-transfection, the cells were lysed for the analysis of protein expression by Western blot or fixed for analysis of protein expression of by immunofluorescence.

4.2.4 Western blot analysis

MDBK cells at 75% confluency were infected with wild-type BAdV-3 at an MOI of 5. At indicated times post-infection, the cells were collected and lysed with 1 mL of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8)) with protease inhibitors (aprotinine, leupeptin, pepstatin A 1 μg/mL each). Proteins from the lysates were separated by 10% SDS PAGE, transferred to the nitrocellulose membrane (Bio-Rad, 162-0115) and probed in Western blot using protein specific antibodies as described (section 3.2.7).

4.2.5 Yeast two-hybrid system

Yeast two-hybrid assay was performed according to the supplier's instruction manual (Clontech, PT3247-1) as described (section 3.2.8).

4.2.6 GST pull-down assay

The assay was performed as described (section 3.2.9) using purified GST, GST-DYNLT1 fusion protein and [^{35}S] labeled *in vitro* transcribed/translated proteins.

4.2.7 Bimolecular fluorescence complementation assay

The assay was performed as described previously (Levin et al., 2009) with minor modifications (section 3.2.10).

4.2.8 Immunoprecipitation/Western blot

293T cells seeded in 60 mm tissue culture plates (Nunc®) were co-transfected with plasmid DNA pcDNA.b100K and pcmyc.DYNLT1 (4 µg each) using the Lipofectamine 2000 reagent (as described in section 4.2.3). At 48 h post-transfection the cells were collected, lysed and immunoprecipitated using anti-100K (N100, C100 or both) sera. The immunoprecipitated proteins were fractionated by 10% SDS-PAGE, transferred to the nitrocellulose membrane (Bio-Rad) and probed in Western blot using monoclonal anti-DYNLT1 (Sigma, D9944).

4.2.9 Immunofluorescence microscopy

MDBK cells were infected with wild type BAdV-3 at 0.5 MOI on 4 well coverslip and processed at 48 h after infection as follows. Similarly transfected Vero cells were also processed at 48 h posttransfection. Cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature, and permeabilized by 0.5% Triton X-100 in PBS for 15 minutes. Cells were stained with primary antibody against 100K and counter stained with Cy2 AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Cat. No.: 111-225-003). Cells were washed in PBS for three times each of 10 minutes and later counter stained with 4',6-diamidino-2-phenylindole (DAPI) followed by three more washes. Later, the coverslips were mounted with Citifluor AF4

mounting media. Images were captured using the different laser lines under laser scanning confocal microscope (Zeiss LSM410).

4.2.10 LC-MS/MS

MDBK cells infected with wild-type BAdV-3 at an MOI of 5 were harvested at 48 h postinfection (p.i.). The cells were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8)) and incubated with anti-100K (N100 and C100 mix) sera coupled to protein A agarose beads were added to the mixture and incubated further at 4 °C for 4 h. After extensive washing, the beads were mixed with 2X SDS-sample buffer, boiled for 2 min, and proteins were separated by 10 % SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Blue R-250 (Sigma). Bands of interest were excised from the SDS gel and sent for liquid chromatography-mass spectrometry (LC-MS/MS) for identifying proteins. Mass spectrometry analysis was performed at the Plant Biotechnology Institute (Saskatoon, Canada) using the robotic workflow of tryptic digestion and chromatography in G-ToF Ultima Mass Spectrometer (Micromass, UK).

4.2.11 Data analysis

Peptides generated by the mass spectrometric analysis of the sample were subjected to NCBI nr database search for the identification of suspected interacting proteins. The database search algorithm programme Mascot from Matrix Science was used to for protein identification. The proteins hits with the scores of above 60 were considered for the current analysis.

4.3 Results

4.3.1 Mass spectrometric identification of cellular proteins interacting with 100K protein of BAdV-3

Protein-protein interactions play an important role in the host-pathogen interaction. In order to identify the cellular proteins interacting with 100K protein during the BAdV-3 infection of MDBK cells, co-immunoprecipation assay using anti-100K (N100 and C100 mix) sera coupled with mass spectrometry analysis was performed. The Mascot search parameter was set to generate the result with p value at <0.05 level of significance. Individual Mascot score above 35 indicated identity or extensive homology. Proteins with two or more peptides identified were considered positive. The search results were manually curated to remove the immunoglobulin chains of *Oryctolagus cuniculus* due to the use of anti-sera raised in rabbit. The LC-MS/MS analysis identified six putative cellular proteins potentially interacting with 100K in BAdV-3 infected cells (Table 4.1).

Since structure and function of dynein light chain particularly in successful virus infections is well documented (Bremner et al., 2009; Lehmann et al., 2009; Lyman and Enquist, 2009), we direct our efforts to the study the interaction of dynein light chain proteins with BAdV-3 100K protein.

4.3.2 Interaction of 100K and DYNLT1 proteins in *vitro*

To verify the results of mass spectrometric analysis and identify the type of dynein light chain interacting with BAdV-3 100K protein, initially yeast two hybrid analysis was performed. Plasmids expressing the DYNLT1 (pGBKT7.DYNLT1), DYNLT3 (pGBKT7.DYNLT3) or DYNLL1 protein (pGBKT7.DYNLL1) fused to binding domain of yeast GAL4 were individually transformed into yeast (*Saccharomyces cerevisiae*) cell AH109 with the plasmid pGADT7.100K

Table 4.2 List of novel suspected protein partners of 100K protein during BAdV-3 infection by LC-MS

Suspected protein partner of 100K	Mascot score	Queries matched
Multi-sensor signal transduction histidine kinase	68	15
Chaperone protein DnaK	67	10
Putative TRAP-type C4-dicarboxylate transport system	63	9
Transcription elongation factor NusA	63	8
ABC transporter, ATPase subunit	62	9
Dynein light chain	67	7

encoding BAdV-3 100K protein fused to DNA activation domain of yeast GAL4. The mixture was plated on the agar plates with selective dropout media. No autoactivation of the reporter genes was observed when individual plasmid DNA was transformed into yeast AH109. All the three co-transformants showed growth on –leucine and –tryptophan plate after 5 days of incubation at 30 °C in humidified chamber (data not shown). There was no growth on the –leucine, –tryptophan and –histidine plates in the cotransformants expressing BAdV-100K fusions and fusion of DYNLT3 or DYNLL1 proteins (data not shown). However, cotransformants expressing DYNLT1 fusion and BAdV-3 100K fusion showed the growth and blue color development on the –leucine, –tryptophan, –histidine, –adenine plates supplemented with the X-alpha-gal (Figure 4.1). These results validated the findings of mass spectrometry analysis and identified the cellular protein DYNLT1 as the interactor of 100K protein of BAdV-3. The interaction between p53 and large T antigen served as positive control for the experiment. The plasmid DNA pair of pGBKT-Lam and pGADT-T served as negative control for the experimental procedure. Similar results were observed using reverse yeast two-hybrid analysis (data not shown).

4.3.3 Interaction of 100K and DYNLT1 *in vivo*

The yeast two-hybrid results were validated by coimmunoprecipitation assay. Plasmid pcmyc.DYNLT1 (expressing N-terminus cmyc epitope tagged DYNLT1 fusion protein) DNA and pcDNA.b100K (expressing the BAdV-3 100K protein) were cotransfected into the 293T cells. At, 48 hours post-transfection, proteins from the lysates of the cells were immunoprecipitated with anti-100K (N100 and C100 mix) sera. The immunoiprecipitated proteins were separated by 10 % SDS-PAGE and probed in Western blot using anti-DYNLT1 MAb. As seen in figure 4.2, two proteins of 12.5 and 14 kDa were observed when proteins from transfected (pcDNA.b100K + pcmyc.DYNLT1) cells were immunoprecipitated with anti-100K (N100 + C100) antibody and

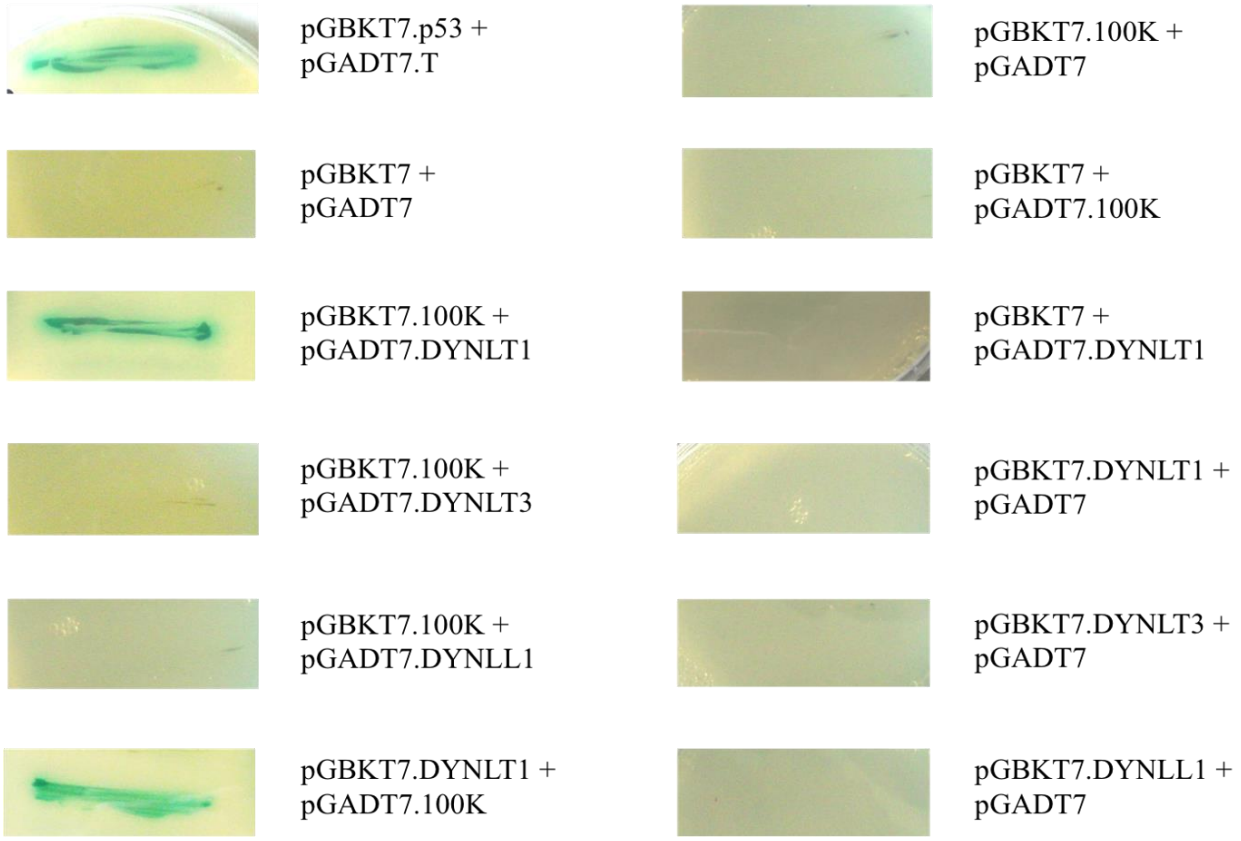

Figure 4.1 Yeast two-hybrid analysis of 100K protein interaction with the dynein light chains.

The yeast cells (AH109) were transformed with the bait and prey plasmids (mentioned on the right side of each photograph) and plated on the –leucine, –tryptophan, –histidine, –adenine plates supplemented with the X-alpha-gal. Plates were incubated at 30 °C for a week and observed for the growth and color development. Plasmid pGBKT7.p53 and pGADT7.T were used as positive controls for the assay.

probed in Western blot with anti-DYNLT1 MAb. No such bands were detected when transfected (pcDNA.b100K+pcmyc.Tctex-1) cells were immunoprecipiated with normal rabbit IgG and probed in Western blot with anti-DYNLT1 antibody. As expected 12.5 kDa protein was detected when transfected cells (pDNA.b100K+pc.myc) were immunoprecipitated with anti-100K (N100 and C100 mix) sera and probed in Western blot using anti-DYNLT1 antibody. No such protein was detected when transfected cells (pDNA.b100K+pc.myc) were immunoprecipiated with normal rabbit IgG and probed in Western blot with anti-DYNLT1 antibody.

4.3.4 Co-localization of 100K protein and DYNLT1 protein

The interaction of 100K protein with the cellular protein DYNLT1 was confirmed by yeast two-hybrid and coimmunoprecipitation assay prompted to probe for the subcellular distribution of 100K and DYNLT1 in the cotransfected cells. Cellular protein DYNLT1 localized to the cytoplasm and nucleus of the transfected cells (Sachdev et al., 2007). BAdV-3 100K protein localized to the cytoplasm of transfected cells (Fig. 4.3).

4.3.5 Identification of region of 100K protein interacting with DYNLT1 protein

To characterize the interaction of the 100K protein of BAdV-3 with cellular protein DYNLT1 an attempt was made to identify the interacting domain of 100K protein. GST pull-down assay was employed for identification of domain of 100K protein interacting with DYNLT1. Initially, four coiled coil regions (121-163aa, 234-280aa, 338-370aa, 463-497aa) were identified in the 100K protein sequence using the COILS server (Lupas et al., 1991). Individual coiled coil region deletions of 100K gene were created in the pcDNA3.100K plasmid background using the standard molecular cloning techniques. The wild type and coiled coil deletions of 100K gene were *in vitro* transcribed and translated in the presence of radiolabelled methionine [^{35}S] and incubated with the GST alone or GST-DYNLT1 in the presence of the glutathione sepharose beads. All the

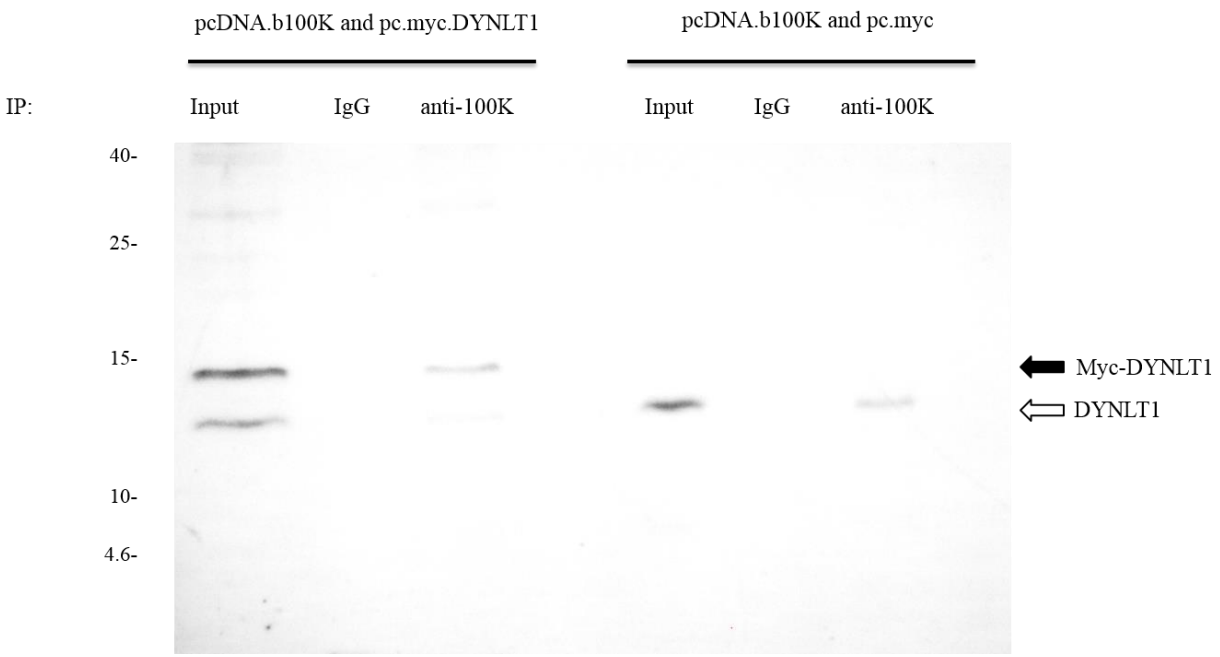

Figure 4.2 Co-immunoprecipitation of 100K and DYNLT1 proteins. Proteins from the lysates of 293T cells transfected with the indicated plasmids DNAs were immunoprecipitated with the anti-100K serum (N100 and C100 mix) or the pre-bleed rabbit serum (IgG). Separated by 15 % SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed using the anti-DYNLT1 MAb and the alkaline phosphate conjugated anti-mouse secondary antibody. Position of molecular weight marker (kDa) is indicated on the left of the panel. Arrows on the right of panel indicates the detected proteins.

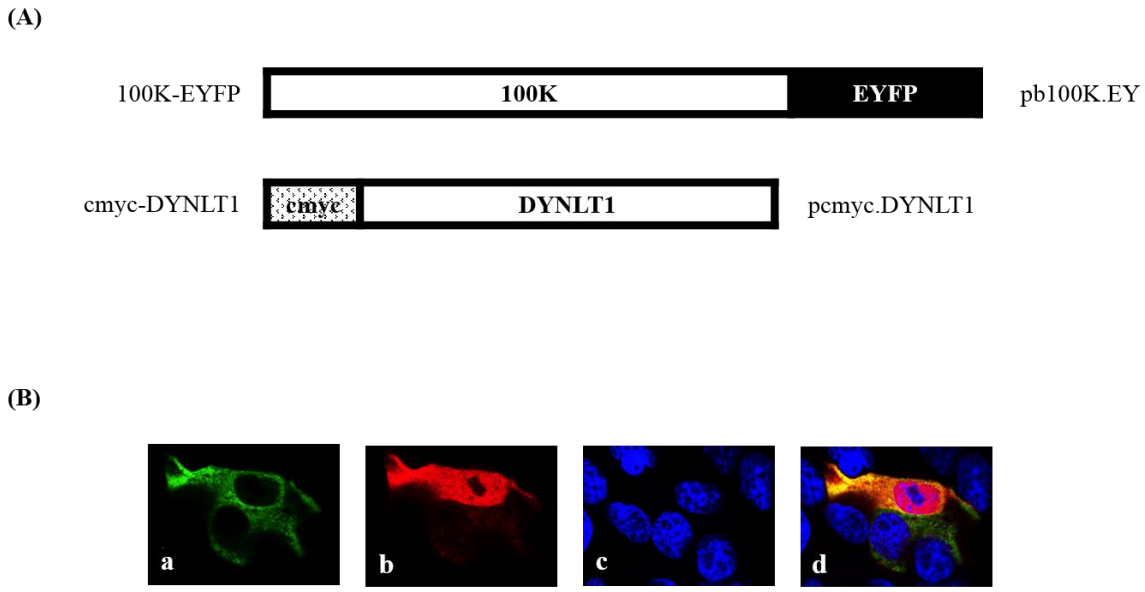

Figure 4.3 Subcellular distribution of 100K protein of BAdV-3 and dynein light chain DYNLT1 protein in cotransfected cells. (A) Schematic representation of the plasmid DNA. The name of the plasmid is depicted on the right of the panel. The name of the protein is depicted on the left of the panel. **(B)** Vero cells were co-transfected with the plasmid DNA encoding EYFP-100K and cmyc-DYNLT1 fusion proteins. At 48 hrs post transfection, the localization of protein was visualized by direct fluorescence (panel a) or indirect fluorescence (panel b) using the anti-cymc MAb. Nuclei were stained with DAPI (panel c). A merge (panel d) of the images is shown.

interaction and absence of role of any coiled coil regions of 100K protein in mediating the interaction with the cellular protein DYNLT1. Interestingly, there was presence of double bands for the *in vitro* translated and transcribed product of 100K protein, which may be due to posttranslational modifications.

Next, the GST pull-down assay was performed with a series of sequential 100K gene deletions from C-terminus. The result suggested amino acids 498-588 of 100K protein are responsible for the interaction with the DYNLT1 protein (Figure 4.5). There was no pull-down of any of the 100K wild-type or deletion proteins with the GST protein alone, implying the specificity of the interaction. The 100K protein interaction with DYNLT1 was confirmed by BiFC assay. There was no green fluorescence visible in the yeast cells transformed with the vectors or interacting region deleted plasmid DNA (Figure 4.6).

4.4 Discussion

Protein-protein interactions between host and pathogen are an important event in the life cycle of viruses. Earlier, a number of adenovirus early proteins and late structural proteins have been reported to interact with different cellular proteins (Wickham et al., 1993; White, 2001; Berk, 2005; Bremner et al., 2009; Arnberg. 2012) and perform important biological functions.

AdV-100K, a late non-structural multifunctional protein has been reported to interact with eIF4G and enhance translation of late viral proteins in HAdV-5 (Tasseron-De Jong et al., 1979; Riley and Flint, 1993; Xi et al., 2005). AdV-100K is also reported to interact with Granzyme H and prevent it from interacting with granzyme B in HAdV-5 preventing apoptosis (Andrade et al., 2001; Waterhouse and Trapani, 2007). Although some cellular factors interacting with 100K have been identified, the existence of additional cellular factors has not been ruled out. Since, 100K

protein is not detected in the mature virion and identified only in the infected cell lysate (Anderson et al., 1973),

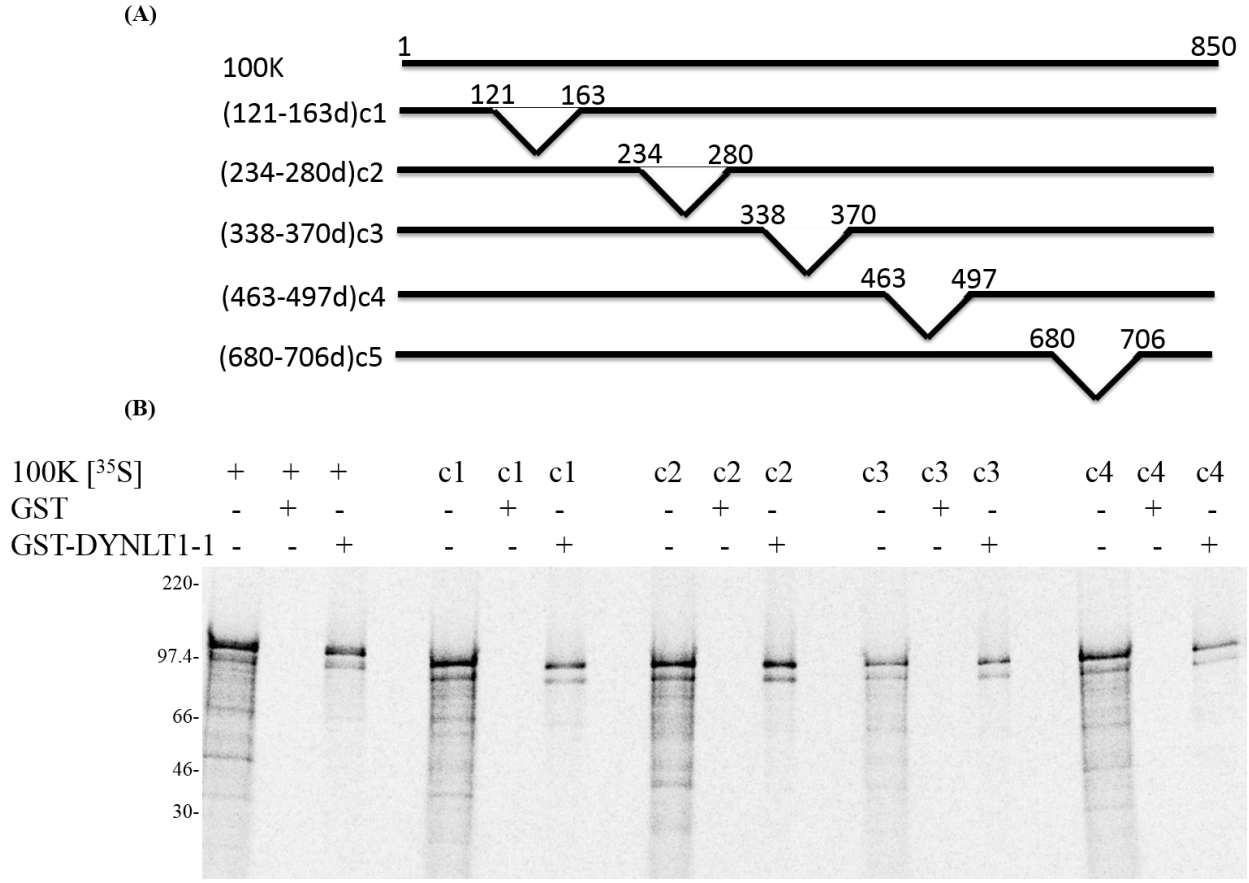

Figure 4.4 Interaction of coiled coils domain deletions of 100K proteins with GST-DYNLT1 protein. (A) Schematic representation of plasmid DNAs. The straight line represents BAdV-3 100K DNA. The number above denotes amino acid position of 100K protein. The name of the protein is depicted on the left of the panel. **(B)** The *in- vitro* transcribed/translated radiolabelled indicated proteins were individually incubated with the purified GST alone or GST-33K fusion protein. The bound protein was separated by 10 % SDS PAGE and visualized by exposure to the phoshorimager screen. Input radiolabelled protein was 5% of the reaction volume. The molecular weights in kDa are indicated on the left side of the figure. The molecular weights in kDa are

indicated on the left of the panel; c1 [pcDNA b100K(121-163d)c1]; c2 [pcD NA.b100K(234-280d)c2]; c3 [pcDNA.b100K(338-370d)c3]; c4 [pcDNA.b100K(463-497d) c4].

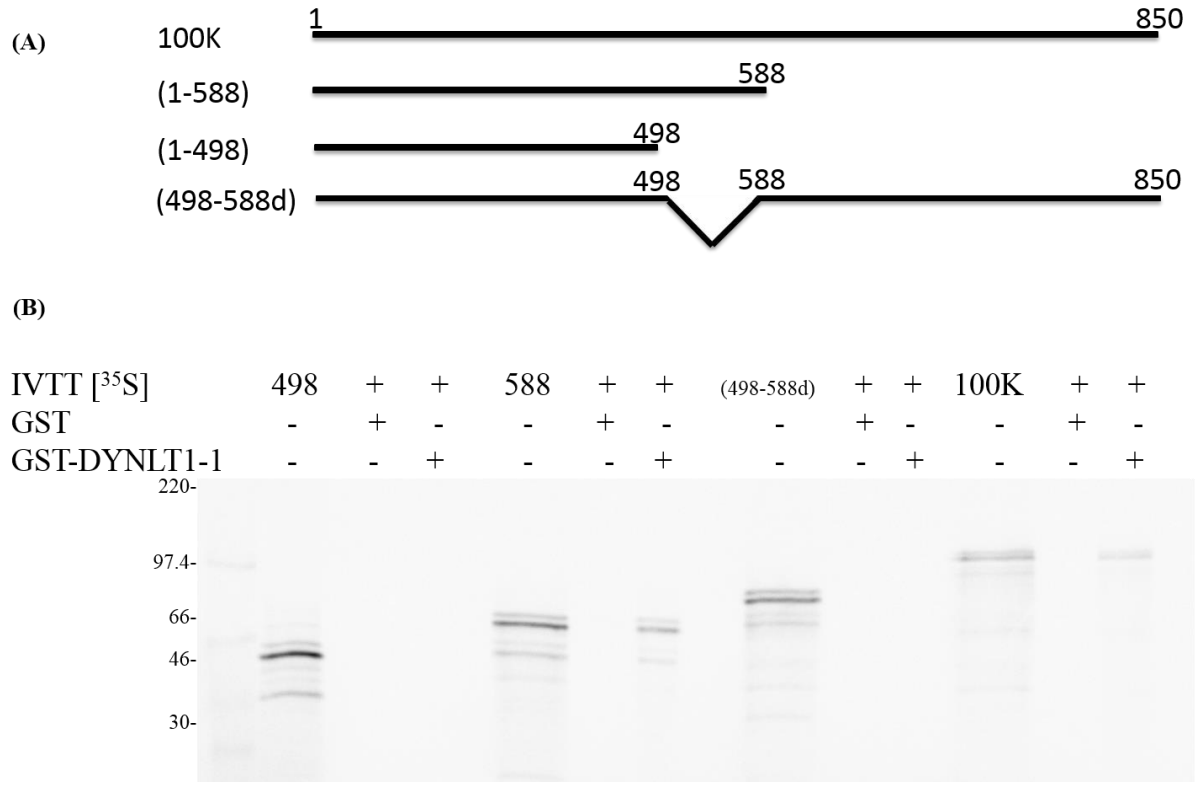

Figure 4.5 Identification of 100K protein region interacting with GST-DYNLT1 protein by GST pull-down assay. (A) Schematic representation of plasmid DNAs. The straight line represents BAdV-3 100K DNA. The number above denotes amino acid position of 100K protein. The name of the protein is depicted on the left of the panel. **(B)** The *in vitro* transcribed/translated radiolabelled indicated proteins were individually incubated with the purified GST alone or GST-33K fusion protein. The bound protein was separated by 10 % SDS PAGE and visualized by exposure to the phoshorimager screen. Input radiolabelled protein was 5% of the reaction volume. The molecular weights in kDa are indicated on the left side of the figure; 498 [pcDNA b100K(1-

498)]; 588 [pcDNA.b100K(1-588)]; (499-588d) [pcDNA.b100K (499-588d)]; 100K [pcDNA.b100K].

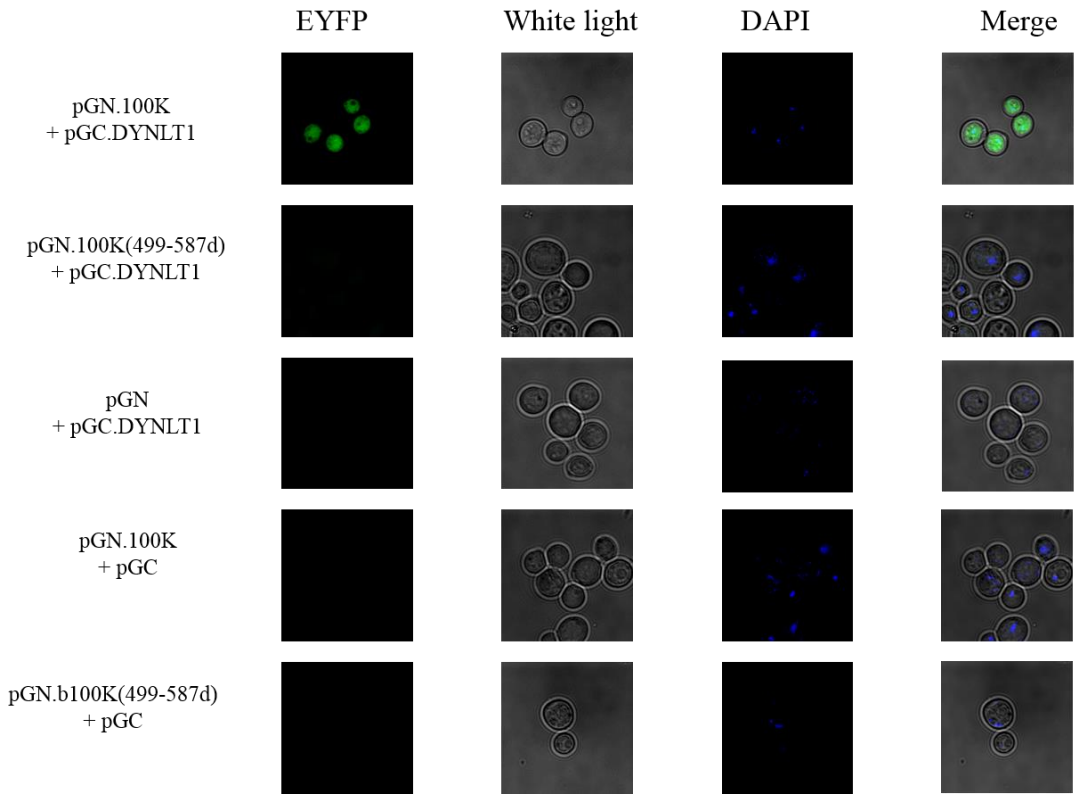

Figure 4.6 Bimolecular fluorescence complementation assay. Yeast cells were co-transformed with the indicated plasmid DNAs plated on the uracil and histidine deficient agar plates and grown at 30° C for 5 days and processed for confocal microscopy for the presence of the green fluorescence using Zeiss LSM 5 laser scanning confocal microscope. Empty vector pGN and pGC were used as negative controls for the self-complementation.

it may interact with the other cellular proteins during the infection process of adenovirus including BAdV-3. Here, we demonstrate that 100K interacts with dynein light chain DYNLT1.

Mass spectrometric analysis of coimmunoprecipitated protein complexes have been employed successfully for identifying the novel cellular proteins interacting with viral proteins (Katoh et al., 2011; Gorai et al., 2012; Shelton et al., 2012). We identified the six novel proteins namely (Table 4.2) coimmunoprecipitated with the 100K protein in BAdV-3 infected MDBK cells. Interestingly, we did not detect the cellular proteins (eIF4G, grazyme B) known to interact with AdV 100K (Andrade et al., 2003; Xi et al., 2004). This could be due to transient nature of the interactions, timing of collection of sample for immunoprecipitation or differences in 100K protein sequences encoded by *Mastadenoviruses*.

Dyneins are composed of four polypeptides (heavy chain, intermediate chain, intermediate light chain and light chain) and are divided into cytoplasmic dyneins or axonemal dyneoins. Cytoplasmic dyneins are molecular motors responsible for the transport of cargo towards the negative end of microtubule (Lo et al., 2007b). The light chain(s) of dynein attach cargo while heavy chain of dynein attached to microtubules help to walk and move cargo along microtubules. The cytoplasmic dynein light chains have been divided into three main types DYNLT, DYNLRB and DYNLL each with two isoforms (Pfister et al., 2005). The cytoplasmic dynein light chain DYNLT1 also known as mouse t-complex testis expressed (Tctex-1) (Lader et al., 1989) or TCTEL1 (Watanabe et al., 1996) is involved in binding (Chuang et al., 2005) with many viral proteins thus helping in transport of different viruses through cytoplasm (Douglas et al., 2004; Vlach et al., 2008; Muller et al., 2010; Schneider et al., 2011).

Since many cellular and viral proteins are found to interact with the dynein protein(s), here we focused on the interaction of 100K with dynein proteins. Since human and bovine DYNLT1

proteins shared 100% identity at amino acid sequence level (data not shown), we used DYNLT1 ORF expressing human DYNLT1 protein. The identified interaction of BAdV-3 100K and DYNLT1 was confirmed by GST-pull down assay. Moreover, co-immunoprecipitation assay confirmed that 100K protein interacted with cellular or plasmid derived and DYNLT11 in transfected cells and co-immunioprecipitation assay. However, initial attempts to detect co-immunoprecipitated DYNLT1 from BAdV-3 infected MDBK cell lysates by Western blot were not successful. Since initial interaction of 100K and DYNLT1 was detected in BAdV-3 infected cells by co-immunoprecipitation/mass spectrometry, it is possible that 100K-DYNLT1 interaction is weak/transient and could not be detected in co-immunoprecipitated proteins from of BAdV-3 infected cell lysates by Western blot analysis.

Although sequence motifs V-S-[K/H]-[T/S]-x-[V/T]-[T/S]-[N/Q]-V (Sugai et al., 2003) and [R/K]-[R/K]-x-x-[R/K] (Mok et al., 2001) in proteins have been reported to be involved in binding to DYNLT1, no consensus motif required for binding to dynein light chain DYNLT1 has been established. Analysis of BAdV-3 100K protein sequence identified DYNLT1 binding motif [R/K]-[R/K]-x-x-[R/K] (Mok et al., 2001) located at amino acid 775-779. However, deletion of amino acid 775-779 did not abolish the binding of BAdV-3 100K to DYNLT1 in GST-pull down assay. Secondly, analysis of BAdV-3 100K protein also identified 4 coiled-coil regions, which have been reported to interact with other proteins (Iacovides et al., 2007). However deletion of each coil-coil region did not alter the binding of BAdV-3 100K to DYNLT1 in GST-pull down assay. Interestingly, analysis of deletions/truncations of 100K protein suggested that amino acid 499-587 of 100K contain the potential DYNLT1 binding region(s).

However, precise location of the DYNLT1 binding region could not be established using additional deletions/ truncation in GST-pull down assay. It is possible that the induced alterations

alter the extended β-strand conformation of 100K required for DYNLT1binding (Merino-Gracia et al., 2011). Alternatively, it is possible that multiple domains located between amino acid 499-587 are involved in binding to DYNLT1, which may be disrupted by deletions/truncations. Interestingly, three domains have been identified in VP26 protein of herpes simplex virus (HSV)-1, which are required for interaction with DYNLT1 (Apcarian et al., 2010).

Although many viral proteins bind dynein light chain (Merino-Gracia et al., 2011) for transport in the dense cytoplasmic environment, interaction of viral protein with dynein light chain also has been reported to be crucial for the activity of rabies viral polymerase and intracellular transport of rabies virus (Tan et al., 2007). Since BAdV-3 100K protein is a nonstructural protein, it is evident that interaction of BAdV-3 100K with DYNLT1 would not be involved in transporting BAdV-3 through cytoplasm during initial stage(s) of virus infection. An earlier report demonstrated the interaction of adenovirus E3 encoded non-structural protein 14.7K with DYNLT1, which may be involved in transport of 14.7K to the site of action (Lukashok et al., 2000). Another report suggested that interaction of E3 non-structural protein 19K with microtubules components may alter the transport of class I major histocompatibility from endoplasmic reticulum to cell surface (Dahllof et al., 1991). It is possible that interaction of 100K with DYNLT1 may be involved in cytoplasmic transport and retention of 100K in the cytoplasm for performing some biological function.

5.0 CLEAVAGE OF BOVINE ADENOVIRUS-3 NON STRUCTURAL 100K PROTEIN IN INFECTED CELLS

5.1 INTRODUCTION

Assembly of progeny adenovirus particles occurs in the nucleus and involves both adenovirus DNA and proteins (Berk, 2007). Although mature adenovirus virions contain only structural proteins, the production of infectious adenovirus virions also requires the involvement of a number of non-structural proteins. Three nonstructural proteins 33K, 22K and 100K encoded by late L4 region of HAdV-5 are involved in different steps of adenovirus replication. Of these, the 100K protein has been shown to be involved in inhibition of host cell protein synthesis (Cuesta et al., 2000), enhancing selective translation of adenovirus late mRNAs (Xi et al., 2004) and preventing apoptosis by inhibiting the activity of granzyme B (Andrade et al., 2001). Moreover, 100K mediates the trimerization and transport of hexon (Hong et al., 2005) for capsid assembly in the nucleus.

Like other viruses (Kohl et al., 1988; Chambers et al., 1990; Gao et al., 1994), production of infectious progeny adenovirus includes a final maturation step involving the cleavage of proteins in assembled capsid by adenovirus protease (Weber, 1995; Mangel et al., 2003). Usually, the inactive form of protease present in immature virions is activated (by viral DNA and pVIc) and cleaves selected virion proteins present in assembled virions leading to the production of infectious progeny virions (Weber, 1976). Mature virions devoid of protease contain DNA but are non-infectious as they unable to penetrate into the cell cytoplasm from the endosome (Greber et al., 1996). Synthesis of an inactive form of adenovirus protease appears to be necessary for avoiding cleavage of precursor proteins before their assembly in virions (Ruzindana-Umunyana et al., 2000; Mangel et al., 2003), thus avoiding abortive infection.

Bovine adenovirus (BAdV)-3 is a non-enveloped icosahedral virion with a linear double stranded viral genome of 34446 bp (Reddy et al., 1998). Analysis of genome sequence and transcriptional map suggest that like human adenovirus (HAdV)-5 (Berk, 2007), BAdV-3 genome is also organized into early, intermediate and late regions. Although genome organization appears similar to HAdV-5, recent studies have identified features distinct to BAdV-3 including organization of late region gene families. A homolog of 100K is also encoded by BAdV-3, which is co-linear with 100K of HAdV-5 and shows 28.3 to 52.1 % identity with 100K proteins of other *Mastadenoviruses* (Reddy et al., 1998). Although cleavage of HAdV-5 100K, a non-structural protein by viral protease has been demonstrated *in vitro*, adenovirus protease specific cleavage of non-structural proteins has not been reported in virus infected/gene transfected cells (Ruzindana-Umunyana et al., 2002). Here, we demonstrate that BAdV-3 100K is cleaved by adenovirus protease in gene transfected/virus infected cells, which does not appear to be essential for BAdV-3 replication.

5.2 MATERIALS AND METHODS

5.2.1 Antibodies

Production of anti-100K (N100 and C100) serum is described in chapter 3.2.2. Production of anti-33K sera (Kulshresthat et al., 2004) and anti-DBP (DNA binding protein) sera (Zhou et al., 2001) has been described. Anti-HA monoclonal antibody (Sigma, H96580), anti-GFP antibody (Sigma, G6539), Cy3 conjugated affinipure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 111-165-003), Cy3 conjugated affinipure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-165-003), Cy2 conjugated affinipure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-225-003) were purchased and used as per

manufacturer's instructions. Monoclonal anti-DYNLT1 antibody was procured from Sigma (D9944).

5.2.2 Cell lines and Viruses

Madin-Darby bovine kidney (MDBK) cells and CRL (cotton rat lung) cells were grown in the minimum essential medium (MEM) (HyClone® Laboratories, Inc., SH30024.01) supplemented with 10 % heat-inactivated gamma irradiated fetal bovine serum (FBS). Vero and 293T cells were propagated in the DMEM supplemented with 10 % FBS, 10 mM HEPES buffer, 0.1 mM NEAA and 50 μg/ml gentamycin. VIDO DT1 (cotton rat lung cells expressing I-SceI protein) cells were propagated in MEM supplemented with 10 % FBS and 50 μg/ml hygromycin B (Clontech Laboratories, Inc., 631309) (Du and Tikoo, 2010). The wild-type and mutant BAdV-3s were propagated and titrated in MDBK cells (Reddy et al., 1998; Kulshreshtha et al., 2004).

5.2.3 Plasmid construction

Construction of plasmids pC.52K (Paterson et al., 2012) and pDR.Nucleolin (Corredor and Archambault, 2009) has been described. The plasmids were constructed using standard procedures. All plasmid constructions were verified by restriction enzyme analysis and DNA sequencing. Details of specific cloning procedures are as follows:

a) Construction of plasmids expressing 100K protein

i) pcDNA.b100K. A 2577 bp DNA fragment amplified by PCR using the primers b100K·F and b100K·R (Table 5.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR product was digested with *Hind*III-*Xba*I and ligated to *Hind*III-*Xba*I digested plasmid pcDNA3.1(+) (Invitrogen, V790-20) creating plasmid pcDNA.b100K.

ii) pHA.b100K. Two complementary oligonucleotides (HA·Link1 and HA·Link2, Table 5.1) designed to code for the HA epitope with four base overhangs compatible with *Hind*III were

annealed to create the linker. HA epitope ecoding linker was ligated to *Hind*III digested plasmid pcDNA.b100K creating plasmid pHA.b100K.

iii) pb100K.EY. A 2580 bp DNA fragment amplified by PCR using primers b100EY·F and b100EY·R (Table 5.1), and plasmid pFBAV304 DNA as a template (Zakhartchouk et al., 1998). The PCR product was digested with *Hind*III-*Age*I and ligated to HindIII-AgeI digested plasmid pEYFP-N1 (Clontech) creating plasmid pb100K.EY.

iv) pHA.b100K.myc.EY. A 2636 bp DNA fragment amplified by PCR using primers B100K·EcoRI·F and B100K·BamHI·R (Table 5.1) and plasmid pFBAV302 (Zakhartchouk et al., 1998). DNA as template. The PCR product was was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 (Clontech, 6006-1) creating plasmid pHA.b100K.myc.EY.

v) pHA.h100K.myc.EY. A2507 bp DNA fragment amplified by PCR using primers H100K·EcoRI·F and H100K·BamHI·R (Table 5.1), and plasmid pHR-5 (Lobanova et al., 2010). DNA as template was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 (Clontech, 6006-1) creating plasmid pHA.h100K.myc.EY.

Plasmid pHA.p100K.myc.EY. A 2600 bp DNA fragment amplified by PCR using primers P100K·EcoRI·F and P100K·BamHI·R (Table 5.1), and plasmid pFPAV200 (Reddy et al., 1999). DNA as a template DNA was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 (Clontech, 6006-1) creating plasmid pHA.p100K.myc.EY.

b) Construction of plasmids expressing BAdV-3 proteins

i) Plasmid pDR.pX. A 269 bp DNA fragment amplified by PCR using primers DRpX·XhoI·F and DRpX·EcoRI·R primers (Table 5.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998).

Table 5.1 List of oligonucleotides

Oligo name	Sequence (5'-3')[a]
b100K·F	AGACCCAAGCTTATGGCAGAGAAAGGCAGTGAAAATC
b100K·R	GGGCCCTCTAGACTACTCTTCTTGCCCTGGCC
HA·Link1	AGCTGAATTCGCCGCCATGGATTACCCATACGACGTACCAGATTA CGCTA
HA·Link2	AGCTTAGCGTAATCTGGTACGTCGTATGGGTAATCCATGGCGGCG AATTC
b100EY·F	GAGCTCAAGCTTATGGCAGAGAAAGGCAGTGAAAATC
b100EY·R	GTGGCGACCGGTCTCTTCTTGCCCTGGCCCTTC
pVIII·EY·F	CATATGAGCAAAGAAATTCCCACACCTTATGTTTGGACC
pVIII·EY·R	ACTGCAGAATTCCGCTATAACCGCTCACAGAGTTGC
DRpX·XhoI·F	ACTGACCTCGAGCTATGAGTCCCCGCGGAAATCTGAC
DRpX·EcoRI·R	ACTGACGAATTCCTATTTGTTGTGGGCCGCCTGAATG
DRHx·XhoI·F	GACGCCCTCGAGTTATGGTCCTACTGCACATCGCC
DRHx·EcoRI·R	TTGTTGGAATTCTTATGTGGTAGCGTTGCCGGCTGAG
B100K·EcoRI·F	AGCTTCGAATTCATGGATTACCCATATGACGTACCAGATTACATG GCAGAGAAAGGCAGTG
B100K·BamHI·R	ACCGGTGGATCCTTCAGATCTTCCTCTGAGATGAGCTTTTGCTCCT CTTCTTGCCCTGGCCCTTC
H100K·EcoRI·F	ACTGACGAATTCATGGATTACCCATATGACGTACCAGATTACATG GAGTCAGTCGAGAAG
H100K·BamHI·R	ACTGACGGATCCTTCAGATCTTCCTCTGAGATGAGCTTTTGCTCCG GTTGGGTCGGCGAACG
P100K·EcoRI·F	ACTGACGAATTCATGGATTACCCATATGACGTACCAGATTACATG GAAGACCAGCACAGCGC
P100K·BamHI·R	ACTGACGGATCCTTCAGATCTTCCTCTGAGATGAGCTTTTGCTCCG AGCCTCGCTGGGGACGAC
BProt·XhoI·F	ACTGACCTCGAGCTATGGGTTCTCGGGAAGAGGAGCTG
BProt·EcoRI·R	CCGGCCGAATTCCTAATTTGTTAACATTTTATCAAAGGCGG
HProt·XhoI·F	ACTGACCTCGAGATATGGGCTCCAGTGAGCAGGAAC
HProt·EcoRI·R	ACTGACGAATTCTTACATGTTTTTCAAGTGAC
PProt·XhoI·F	TCAGATCTCGAGATATGGGCAGCACCGAGGACG
PProt·EcoRI·R	ACTGCAGAATTCTTATTGCATATCGAGCGCTTTGTGC
33K·KpnI·F	AAGCTTGGTACCATGAAACCCCGCAGCATGTC

Table 5.1 cont.

Oligo name	Sequence (5'-3')[a]
33K·BamHI·R	GCCATGGGATCCAAGGCGGGTCCGGATTCGTCC
100K·M·F1	GCTTGTGTCATCACTGAAGAAAAAGTTTTAGCCC
100EY·706·R1	GGTCTGAGGGTCCAGGTATACTGCGTGCGCCTTTTTAAGGAGAAAGTC
100EY·706·F2	TTCTCCTTAAAAAGGCGCACGCAGTATACCTGGACCCTCAGACCGGC
100EY·742·R1	AGCTGTTGGCCCTCCGAGGGCGCTAGCGCGGAAGGCCCGGCTGCCGAC
100EY·742·F2	GGCAGCCGGGCCTTCCGCGCTAGCGCCCTCGGAGGGCCAACAGCTGC
100EY·746·R1	TGTGGCGGCAGCTGTTGGCGCTGCTAGCCCTGAGCCGCGGAAGGCCCGG
100EY·746·F2	GGCCTTCCGCGGCTCAGGGCTAGCAGCGCCAACAGCTGCCGCCACAGAC
100EY·783·R1	CCGTCAGGGGGAAGTCGACCTGCCGCTCGGATATGTCTTCCAGG
100EY·783·F2	CCTGGAAGACATATCCGAGCGGCAGGTCGACTTCCCCCTGACGG
100b2.EY EcoRI·F	AGCTTCGAATTCGCCGCCATGGGCCTCGGAGGGCCAACAGC
100b2.EY BamHI·R	ACCGGTGGATCCGCCTCTTCTTGCCCTGGCCC
100b3.EY EcoRI·F	AGCTTCGAATTCGCCGCCATGGGAAGGCTTCCCCCTGACGGACG
p100b1.EY·R	GGTGGCGACCGGTGAGCCGCGGAAGGCCCGGCTGCCG
CAT.SbfI.F	CCTGCAGGTACCCAATACTCTTCATC
CAT,SbfI.R	CCTGCAGGTAAGCTGCAATAAACAAG
b100K·742·F1	GCTTGTGTCATCACTGAAGAAAAAGTGCTAGCCCAATTGCATGAAATTAAAAAAGC
b100K·742·R1	GGCAGCTGTTGGCCCTCCGAAAGCTGAGGCGCGGAAGGCCCGGCTGCCGAC
b100K·742·F2	TCGGCAGCCGGGCCTTCCGCGCCTCAGCTTTCGGAGGGCCAACAGCTGCCG
b100K·742·R2	AGGGCGAGGGCGCGCCGCGCTGGCACTCCGGCGGCTCTTTTTGC
100C1·F	AGACCCAAGCTTCCACCATGGAAGGTAGTAGCTTCTCTG
100C1·R	TCCTTTGCTAGCCTCTTCTTGCCCTGGCCCTTC

[a] Restriction enzyme sites are underlined.

DNA as template was digested with *Xho*I-*Eco*RI and ligated to *Xho*I-*Eco*RI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR·pX.

ii) Plasmid pDR.Hexon. A 2735 bp DNA fragment amplified by PCR using primers DRHx·XhoI·F and DRHx·EcoRI·R (Table 5.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998). DNA as a template was digested with *Xho*I-*Eco*RI enzymes and ligated to *Xho*I-*Eco*RI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.Hexon.

iii) Plasmid pDR.33K. A 863 bp DNA fragment amplified by PCR using primers 33K·KpnI·F and 33K·BamHI·R (Table 5.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998). DNA as a template was digested with *Kpn*I-*Bam*HI enzymes and ligated to *Kpn*I-*Bam*HI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.33K.

iv) Plasmid pVIII.EY. PCR was performed to amplify 664 bp product using pVIII·EY·F and pVIII·EY·R primers (Table 5.1) using plasmid pFBAV302 (Zakhartchouk et al., 1998) as template DNA. The PCR product was digested with *Eco*RI enzyme and cloned into plasmid pEYFP-N1 (Clontech, 6006-1) at the *Age*I (blunt end filled-in)-*Eco*RI sites to create pVIII.EY.

c) Construction of plasmids expressing protease protein

i) Plasmid pDR.bProt. A 641 bp DNA fragment (coding BAdV-3 protease gene) amplified by PCR using primers BProt·XhoI·F and BProt·EcoRI·R (Table 5.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998). DNA as a template DNA was digested with *Xho*I-*Eco*RI and ligated to *Xho*I-*Eco*RI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.bProt.

ii) Plasmid pDR.hProt. A 641 bp DNA fragment (coding HAdV-5 protease gene) using primers HProt·XhoI·F and HProt·EcoRI·R (Table 5.1), and plasmid pHR-5 (Lobanova et al., 2010).

DNA as a template was digested with *Xho*I-*Eco*RI and ligated to *Xho*I-*Eco*RI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.hProt.

iii) Plasmid pDR.pProt. A 641 bp DNA fragment (coding PAdV-3 protease gene) using primers PProt·XhoI·F and PProt·EcoRI·R (Table 5.1), and plasmid pFPAV200 (Reddy et al., 1999). DNA as a template was digested with *Xho*I-*Eco*RI enzymes and ligated to *Xho*I-*Eco*RI digested plasmid pDsRed-monomer C1 (Clontech, 632466) creating plasmid pDR.pProt.

d) Construction of plasmids expressing mutant 100K

i) Plasmid pbG706A.G708A. A 111 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and100EY·706·R1 and (Table 5.1), and plasmid pcDNA.b100K DNA as a template. Similarly, a 460 bp DNA fragment was amplified by PCR using primers 100EY·706·F2 and b100EY·R (Table 5.1) and plasmid pcDNA.b100K as a template. Finally, the two PCR products were annealed and a 528 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and b100EY·R (Table 5.1). The final PCR product was digested with *Age*I and ligated to *Afe*I-*Age*I digested plasmid pb100K.EY generating plasmid pbG706A.G708A.

ii) Plasmid pbG742A.G744A. A 216 bp DNA fragment amplified by PCR using primers 100K·M·F1 and 100EY·742·R1 (Table 5.1) and plasmid pcDNA.b100K DNA as a template. Similarly, a 356 bp DNA fragment was amplified using primers 100EY·742·F2 and b100EY·R (Table 5.1) and plasmid pcDNA.b100K DNA as a template. Finally, the two PCR products were annealed and 528 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and b100EY·R (Table 5.1) The final PCR product was digested with *Age*I and ligated to *Afe*I-*Age*I digested plasmid pb100K.EY generating plasmid pbG742A.G744A.

iii) Plasmid pbG746A.G747A. A 225 bp DNA fragment amplified by PCR using primer 100K·M·F1 and 100EY·746·R1 (Table 5.1) and plasmid pcDNA.b100K DNA as a template.

Similarly, a 348 bp DNA fragment was amplified by PCR using primers 100EY·746·F2 and b100EY·R (Table 5.1), and plasmid pcDNA.b100K DNA as a template PCR. Finally, the two PCR products were annealed and a 528 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and b100EY·R (Table 5.1). The final PCR product was digested with *Age*I and ligated to *Afe*I-*Age*I digested plasmid pb100K.EY generating plasmid pbG746A.G747A.

iv) Plasmid pbG783A.G784A. A 338 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and 100EY·783·R1 (Table 5.1), and plasmid pcDNA.b100K DNA as a template. Similarly, a 234 bp DNA fragment was amplified by PCR using primers 100EY·783·F2 and b100EY·R (Table 5.1), and plasmid pcDNA.b100K DNA as a template DNA. Finally, the two PCR products were annealed and a 528 bp DNA fragment was amplified using primers 100K·M·F1-b100EY·R (Table 5.1). The final PCR product was digested with *Age*I and ligated to *Afe*I-*Age*I digested plasmid pb100K.EY generating plasmid pbG783A.G784A.

v) Plasmid pbG742A.G783A. A 338 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and 100EY·783·R1 (Table 5.1), and plasmid pbG742A.G744A DNA as a template. Similarly, a 234 bp DNA fragment was amplified by PCR using primers 100EY·783·F2 and b100EY·R (Table 5.1), and plasmid pbG742A.G744A DNA as a template DNA. Finally, the two PCR products were annealed and a 528 bp DNA fragment was amplified using primers 100K·M·F1-b100EY·R (Table 5.1). The final PCR product was digested with *Age*I and ligated to *Afe*I-*Age*I digested plasmid pbG742A.G744A generating plasmid pbG742A.G783A.

e) Construction of plasmids expressing nuclear localizing mutant 100K

i) Plasmid p100b1.EY. A 2253 bp DNA fragment (coding 1-743 amino acids of BAdV-3 100K protein) amplified by PCR using primers b100EY·F and 100b1.EY·R (Table 5.1), and plasmid

pcDNA.b100K DNA as a template was digested with *Hind*III-*Age*I and ligated to *Hind*III-*Age*I digested plasmid pEYFP-N1 (Clontech, 6006-1) creating plasmid p100b1.EY.

ii) Plasmid p100b2.EY. A 356 bp DNA fragment amplified by PCR using primers 100b2.EY EcoRI·F and 100b2.EY BamHI·R (Table 5.1), and plasmid pcDNA.b100K DNA as a template with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 creating plasmid p100b2.EY.

iii) Plasmid p100b3.EY. A 233 bp DNA fragment amplified by PCR using primers 100b3.EY EcoRI·F and 100b2.EY BamHI·R (Table 5.1), and plasmid pcDNA.b100K DNA as a template was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 (Clontech, 6006-1) creating plasmid p100b3.EY.

iv) Plasmid p100.C1. A 287 bp DNA fragment (coding 766-850 amino acids of BAdV-3 100K protein) amplified by PCR using primers 100C1·F and 100C1·R (Table 5.1), and plasmid pHA.b100K DNA as a template was digested with *Hind*III-*Nhe*I and ligated to *Hind*III-*Nhe*I digested plasmid pGFPβGal (Wu et al., 2004) creating plasmid p100.C1.

v) Plasmid p100.C2. A 218 bp DNA fragment (coding 766-850 amino acids of BAdV-3 100K protein with 789-811 amino acid coding deletion) amplified by PCR using primers 100C1·F and 100C1·R (Table 5.1), and plasmid pHA.b100K DNA as a template was digested with *Hind*III-*Nhe*I and ligated to *Hind*III-*Nhe*I digested plasmid pGFPβGal (Wu et al., 2004) creating plasmid p100.C2.

5.2.4 Western blot analysis

The cells were infected with wild-type BAdV-3 at a MOI of 5 or transfected with plasmid DNAs (10 μg /well). At indicated times post-infection or transfection, the cells were collected,

lysed and proteins analyzed by Western blot using protein specific antibodies as described (Kulshrestha et al., 2004).

5.2.5 Confocal microscopy

The cells ($2x10^5$ cells/ well) grown into 4-chambered poly-L-lysine-coated glass coverslips were infected with wild-type BAdV-3 at a MOI of 1 or transfected with plasmid DNA (1 µg/well). At 48 hrs post infection or transfection, the cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature before permeabilizing with 0.5% Triton X-100 in PBS for 15 min. The permeabilized cells were incubated with protein specific primary antibody for 60 min at 25 °C followed by specific conjugated secondary antibody for 60 min at 25 °C. Finally, the cells were washed in PBS three times (10 min/wash) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with Citifluor AF4 mounting media. Images were captured using the different laser lines under laser scanning confocal microscope (Zeiss LSM410).

5.2.6 Isolation of protease cleavage BAdV-3 mutants

To isolate mutant BAdV-3s, we constructed full length BAdV-3 plasmids containing mutant BAdV-3 genomic DNA as described (Chartier et al., 1996).

i) Plasmid pUC304aΔ100K. A 9962 bp EcoRV-*Nde*I DNA fragment of plasmid pUC304a+ (Du and Tikoo, 2010) was isolated and digested with *Sal*I. Finally, a 6477 bp *Eco*RV-*Sal*I DNA fragment was isolated and ligated to *Sma*I-*Sal*I digested plasmid pUC18 creating plasmid pUC18.b100K. Next, an 894 bp DNA fragment was amplified by PCR using primers (CAT·SbfI·F and CAT·SbfI·R) and plasmid pBAC-lacZ (Addgene plasmid 13422) DNA as a template, and ligated to *Nhe*I-*Afe*I (blunt end repaired by T4 polymerase) digested plasmid pUC18.b100K to create plasmid pUC18.b100K.CAT.SbfI. A 4941 bp *Bam*HI fragment of plasmid DNA pUC18.b100K.CAT.SbfI was isolated and recombined with *Mfe*I digested

plasmid DNA pUC304a+ (Du and Tikoo, 2010) in *E. coli* BJ5183 to generate plasmid pUC304a.CAT.SbfIΔ100K. Finally, plasmid pUC304a.CAT.SbfIΔ100K was digested with *Sbf*I and large fragment was self-ligated to generate plasmid pUC304aΔ100K.

ii) *Plasmid pFBAV742*. A 219 bp DNA fragment was amplified by PCR using primers b100K·742·F1 and b100K·742·R1, and plasmid pUC18.b100K DNA as a template. Similarly, a 631 bp DNA fragment was amplified by PCR using primers b100K·742·F2 and b100K·742·R2, and plasmid pUC18.b100K DNA as a template. Both PCR products were annealed and used as DNA template for primers b100K·742·F1-b100K·742·R2 to amplify 800 bp DNA fragment by PCR. The final PCR product was digested with *Asc*I and cloned in to *Afe*I-*Asc*I digested plasmid pUC18.b100K to create plasmid pUC18100K.G742A/G744A. A 6163 bp BglII-SalI DNA fragment of plasmid pUC18100K.G742A/G744A was isolated and recombined with *Sbf*I linearized plasmid pUC304aΔ100K DNA in E. coli BJ5183 creating plasmid pFBAV742.

iii) *Plasmid pFBAV742/782*. A 344 bp DNA fragment was amplified by PCR using primers 100K·M·F1 and 100EY·783·R1, and plasmid pUC18100K.G742A/G744A DNA as a template. Similarly, a 509 bp DNA fragment was amplified by PCR using primers 100EY·783·F2 and b100K·742·R2, and plasmid pUC18100K.G742A/G744A DNA as a template. Both PCR products were annealed and used as DNA template for primers b100K·742·F1-b100K·742·R2 to amplify 800 bp DNA fragment by PCR. The final PCR product was digested with *Asc*I and cloned in to *Afe*I-*Asc*I digested plasmid pUC18.b100K to create plasmid pUC18100K.G742A/G783A. A 6163 bp *Bgl*II-*Sal*I DNA fragment of plasmid pUC18100K.G742A/G783A was isolated and recombined with *Sbf*I linearized plasmid pUC304aΔ100K DNA in *E. coli* BJ5183 creating plasmid pFBAV742/783.

To isolate mutant BAdV-3, the VIDO DT1 cells were transfected with individual recombinant full length plasmid DNAs (10 μg/well) using Lipofectamine^{TM} 2000 (Life Technologies, 11668-019) as described (Reddy et al., 1999; Du and Tikoo, 2010).

5.2.7 Virus infected cell global protein synthesis assay

Monolayers of MDBK cells in 6 well plates were mock infected or infected with wild type BAV304a or mutant virus BAV742/783 at MOI of 5. At different times post infection, the cells were starved in media without methionine for 2 hrs before labeling with 100 μCi of [^{35}S] methionine for 10 minutes. The radiolabelled cells were collected and lysed with RIPA buffer containing protease inhibitor cocktail. Proteins from radiolabelled cell lysates were separated by 10% SDS-PAGE. The gel was fixed, exposed to a phosphor screen (Kodak) overnight and visualized on a Molecular Imager FX using Quantity One software (Bio-Rad).

5.3 RESULTS

5.3.1 Co-expression of 100K protein with other late proteins of BAdV-3

Earlier (Chapter 3), we demonstrated that 100K protein is predominantly localized in the cytoplasm of transfected cells and interaction of 100K and 33K does not alter the localization of 100K in co-transfected cells (Fig. 3.5). To determine the role of other BAdV-3 proteins in nuclear localization of 100K, we performed co-transfection experiments. As seen in figure 5.1 (A, B) 100K-EYFP was exclusively localized to the cytoplasm of cells transfected with plasmid pb100K.EY (panel g). Similarly, 100K–EYFP fusion protein was exclusively localized in the cytoplasm of the cells co-transfected with plasmid pb100.EY+ pc.52K DNAs (panel a), pHA.b100K+pEY.VIII (panel b), pb100.EY+pDR.X (panel c) or pb100.EY+pDR.hexon (panels d). The DsRed–protease fusion protein localized to both cytoplasm and nucleus of the cells

transfected with plasmid pDr.bProt DNA (panel e). In contrast, co-transfection of cells with plasmid pb100K.EY + pDr.bProt DNAs resulted in the localization of recombinant b100K.EY protein in the nucleus of transfected cells without any alteration in the subcellular distribution of recombinant DsRed.bProt fusion protein in co-transfected cells (panels e1 and f1). These results suggest a role of the protease of BAdV-3 in subcellular distribution of 100K protein in cells.

5.3.2 Cleavage of BAdV-3 100K in transfected cells

To further confirm the localization of 100K in cells expressing protease, the 293T cells were co-transfected with plasmid pb100K.EY and pDR.bProt DNAs. At 48 hrs post transfection, proteins from the lysates of transfected cells were separated by 12 % SDS-PAGE and analyzed by Western blot using antibodies recognizing the N-terminus (N100) or C-terminus (C100) of BAdV-3 100K protein. As seen in figure 5.2, N100 (panel B) sera detected proteins of 130 kDa in cells co-transfected with plasmid pb100K.EY and pDsRed-C1 DNAs, and protein of 100 kDa in cells transfected with plasmid pb100K.EY and pDR.bProt. Similarly, C100 (panel C) sera detected proteins of 130 kDa in cells co-transfected with plasmid pb100K.EY + pDsRed-C1. In contrast, C100 sera detected a protein of 40 kDa in cells co-transfected with plasmid pb100K.EY + pDR.bProt. No such proteins could be detected in cells co-transfected with plasmid pb100K.EY + pDsRed-C1.

5.3.3 Cleavage of 100K in BAdV-3 infected cells

To determine if 100K protein is also cleaved in BAdV-3 infected cells, the MDBK cells were infected with BAdV-3 at an MOI of 5. At 48 hrs post infection, the proteins from the lysates of the infected cells were separated by 15 % SDS-PAGE and probed in Western blot using anti-100K sera. As seen in figure 5.3 N100K sera detected the proteins of the 130 kDa (panel A) in BAdV-3

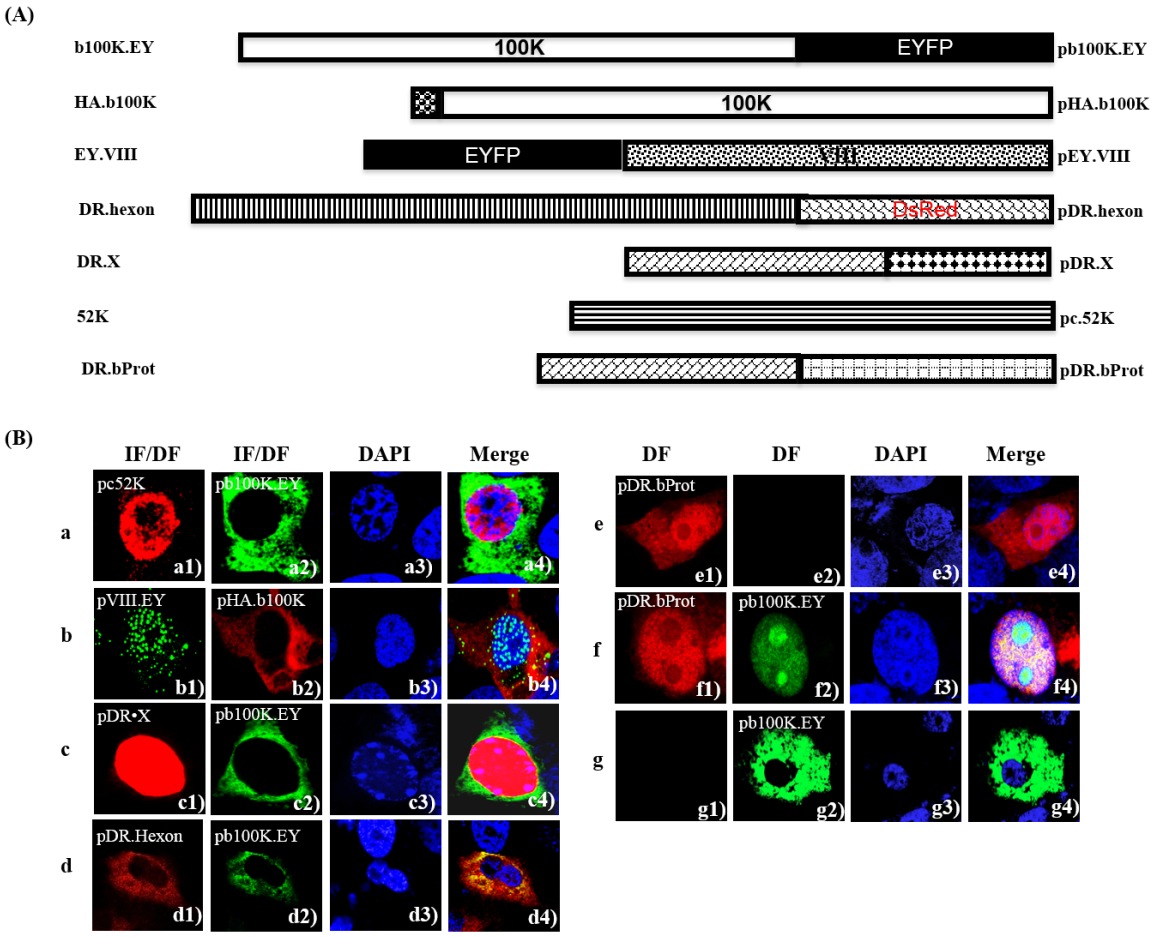

Figure 5.1 Subcellular distribution of BAdV-3 100K protein in co-transfected cells. (A) Schematic diagram of plasmid DNAs. The origin of DNA BAdV-3 100K ( ), Hexon ( ), 52K ( ), X ( ), protease ( ) and EYFP ( ), HA tag ( ) DsRed ( ) is depicted. **(B)** Vero cells cotransfected with plasmid DNAs pc.52K + pb100.EY (panel a), pVIII.EY + pHA.b100K (panel b), pDR.pX + pb100K.EY (panel c), pDR.Hexon + pb100K.EY (panel d), pDR.bProt + pb100K.EY (panel f), or transfected with plasmid pDR.bProt (panel e) or pb100K.EY (panel g). The localization of protein was visualized by direct fluorescence (panels a2, b1, c, d, e, f, g) or indirect immune fluorescence (panels a1, b2) using anti-52K serum (panel a1) or anti-HA monoclonal antibody (panel b2). Nuclei were stained with DAPI in each panel. A merge of the images is shown.

infected cells. In contrast, the C100K sera detected a protein of 15 kDa in BAdV-3 infected cells (panel B). No such proteins could be detected in the mock-infected MDBK cells (panel A, B).

5.3.4 Analysis of BAdV-3 100K protein sequence for potential protease cleavage sites

Based on the cleavage of adenovirus structural proteins (Mangel et al., 2003) by adenovirus encoded protease, at least two consensus protease cleavage sequences (M/I/L)*X*G*X*-G and (M/I/L)*X*GG-*X* have been identified (Diouri et al., 1996). Consensus adenoviral protease cleavage sequences are composed of glycine residues essential for the cleavage of substrate proteins (Ruzindana-Umunyana et al., 2002). Analysis of amino acid sequence of BAdV-3 100K identified four potential protease cleavage sites (Table 5.2).

5.3.5 Mutational analysis of potential protease cleavage site(s) of BAdV-3 100K protein

To further characterize the cleavage of 100K protein by BAdV-3 protease, we constructed plasmids (pbG706A.G708A, pbG742A.G744A, pbG746A.G747A, pbG783A.G784A and pbG742A.G783A) encoding mutant 100K in which the essential glycine residues of potential protease cleavage sites of 100K were substituted with alanine residues (Fig. 5.4). Cleavage of mutant 100K proteins by adenovirus protease was analyzed by Western blot of the lysates of the cells co-transfected with plasmid pDR.bProt and individual plasmid DNAs encoding wild-type or mutant 100K protein. As seen in figure 5.4, anti-GFP antibody detected a major protein of 40 kDa and a minor protein of 36 kDa in the cells expressing b100K.EY fusion protein and protease. Similar proteins were detected in cells co-expressing protease and mutant b100K.706/708 or mutant b100K.746/747 proteins using anti-GFP antibody (Fig. 5.4). Moreover, cleavage of 100K appeared efficient in cells expressing protease as no uncleaved 100K protein could be detected (Fig. 5.4). In contrast, anti-GFP antibody detected only 36 kDa protein in cells expressing protease and mutant b100K.742/744 protein (Fig. 5.4). In addition, cleavage of 100K appeared inefficient

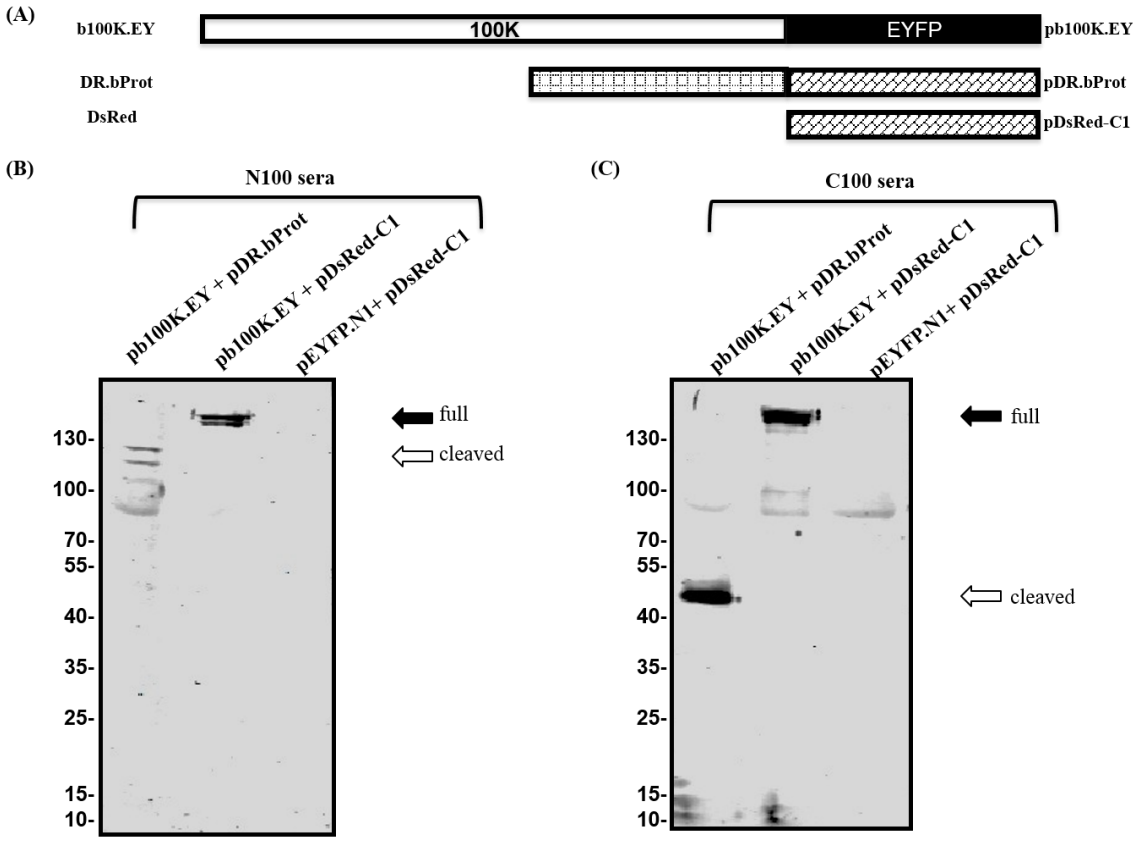

Figure 5.2 Cleavage of BAdV-3 100K protein in transfected cells. (A) Schematic diagram of plasmid DNAs. The origin of BAdV-3 100K ( ), BAdV-3 protease ( ) and DsRed ( ) DNA is depicted. **(B)** Proteins from the lysates of 293T cells co-transfected with the indicated plasmid were fractionated by 15 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-100K sera: N100 (panel B) or C100 (panel C). Filled arrows indicate mature b100K-EY fusion protein and empty arrows indicates the cleaved product.

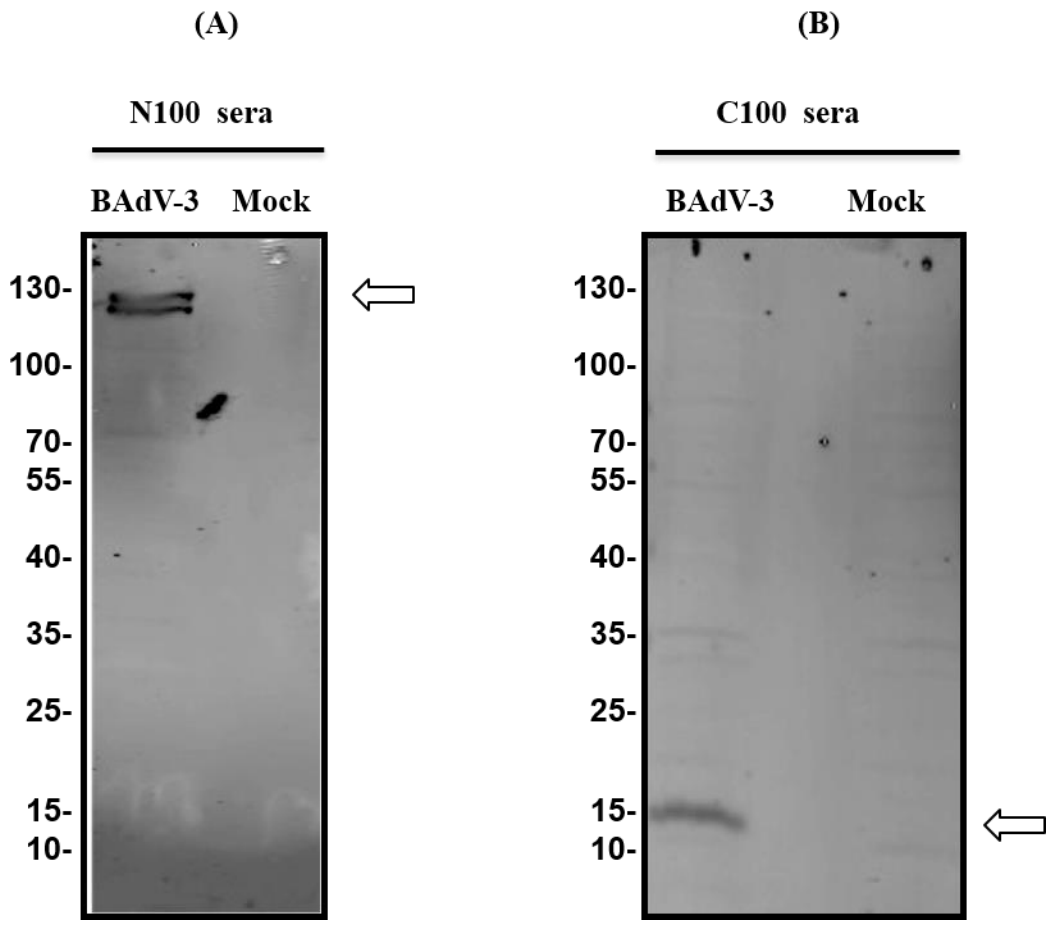

Figure 5.3 Cleavage of 100K protein in BAdV-3 infected cells. Proteins from the lysate of BAdV-3 infected or mock infected MDBK cells harvested at 48 hrs post infection (panel A,B) were separated by 15% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-100K specific sera; N100 (panel A) or C100 (panel B) antibodies. The detected proteins are indicated by arrows.

Table 5.2 List of potential protease cleavage sequences in 100K protein of BAdV-3

Potential cleavage sequence	Mutations
704-KK**G**H**G**V-709	G706A, G708A
740-FR**G**S**G**L-745	G742A, G744A
743-SGL**GG**P-748	G746A, G747A
781-IR**GG**GR-786	G783A, G784A

as uncleaved mutant 100K could be detected in these cells (Fig. 5.4). Only 40 kDa protein could be detected in the cell lysates of the cells cotransfected with the plasmid DNA b100K.783/784 and pDR.bProt (Fig. 5.4). No cleaved fragments can be detected from the cell lysate cotransfected with the plasmid DNA b100K.742/783 and pDR.bProt (Fig. 5.4).

To further characterize the cleavage of BAdV-3 100K, the subcellular localization of protease cleavage site mutant 100K proteins was analyzed in cells co-expressing BAdV-3 protease by confocal microscopy. As seen in figure 5.5 wild-type (panel a) or single protease cleavage site mutant 100K proteins (panel b, c) localized predominantly in the nucleus of transfected cells co expressing protease. In contrast, double protease cleavage site mutant 100K localizes predominantly to the cytoplasm of cells co-expressing protease (panel d).

To confirm which cleavage fragment of 100K localized to nucleus, we constructed three plasmids encoding mutant 100K individually fused in frame to the gene encoding EYFP (Fig. 5.6A). The Vero cells were co-transfected with individual 100K mutant plasmid DNA and plasmid p100b2.EY DNA, and examined by direct fluorescence. As seen in figure 5.6B, EYFP fused to either amino acid 744-850 (cleavage fragment 1; panel b) or amino acid 785-850 (cleavage fragment 2; panel c) of 100K is predominantly localized to the nucleus of transfected cells. However, EYFP fused to amino acids 1-743 of 100K protein localized to the cytoplasm of the transfected cells (panel a).

Taken together, these results suggest that adenoviral protease recognizes two protease cleavage sites (amino acids 740-745 & 781-786) at the C-terminus portion of 100K protein of the BAdV-3. Moreover, our results suggest that cleaved C-terminus of 100K localizes to the nucleus.

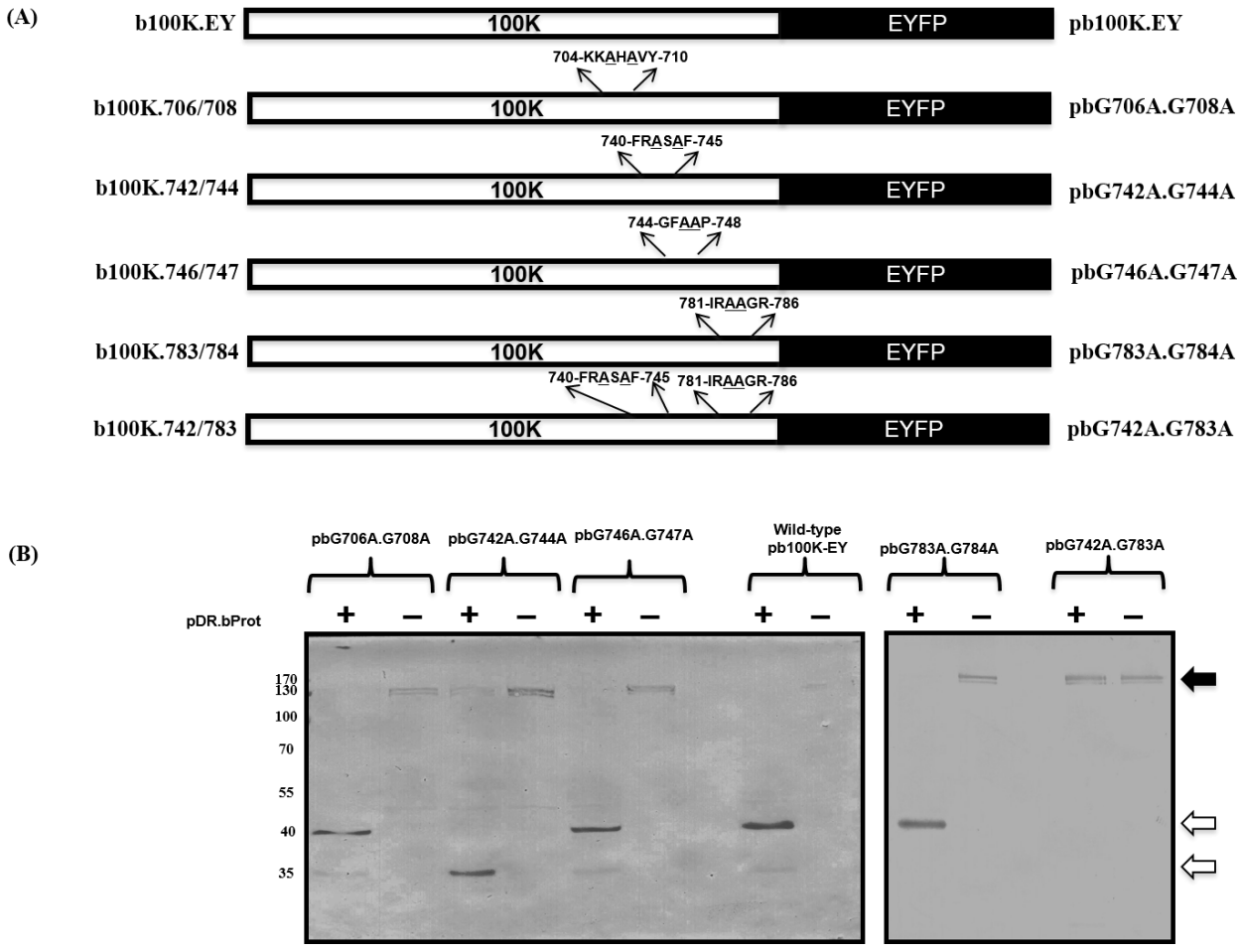

Figure 5.4 Expression of BAdV-3 100K cleavage of mutant protein in co-transfected cells. **(A)** Schematic diagram of plasmid DNAs. The origin of DNAs is depicted. The substituted alanines for glycines in predicted protease cleavage sites are underlined. The name of the plasmid is on the right side of the panel. The name of the protein is on the left side of the panel. **(B)** Proteins from the lysates of indicated plasmid DNA transfected 293T cells harvested at 48 hrs post transfection were separated by 15% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-GFP monoclonal antibody. Filled arrows indicate mature 100K-EY fusion protein and empty arrows indicates the cleaved 100K-EY fusion protein.

5.3.6 C-terminus of 100K contain nuclear localization signal

Analysis of BAdV-3 100K protein sequence by PSORT II computer program (Horton et al., 2007) identified a potential bipartite nuclear localization signal at amino acids 789 to 811. To determine if amino acids 789-811 containing basic residues are important for nuclear transport, amino acids 766-850 or 766-850 (containing deletion of amino acid 789-811) were individually fused in-frame to the N-terminus of a green fluorescent protein-β galactosidase (GFPβGal) fusion to produce chimeric bNLS-GFPβGal and bNLSdel-GFPβGal. As seen in figure 5.7B, bNLS-GFPβGl localized predominantly in the nucleus of transfected cells. In contrast, similar to GFPβGal (Wu et al., 2004) bNLSdel-GFPβGal fusion protein localized predominantly to the cytoplasm of the transfected cells.

To determine if 100K protein can interact with the importins, we performed the GST-pull down assay. GST alone or GST fused to importin α1 α3, α7 or β1 was incubated individually with *in vitro* transcribed/translated [^{35}S] labeled 100K. The bound proteins were separated by 10 % SDS-PAGE and visualized by autoradiography. As seen in figure 5.7C *in vitro* translated 100K interacted with GST-importin α3 fusion protein. No interaction was detected between in vitro synthesized 100K with GST alone or GST fused to importin α1, α7 or β1.

5.3.7 Cleavage of 100K protein in other *Mastadenoviruses*

To determine if the cleavage of 100K protein is conserved in other members of *Mastadenoviruses,* we constructed plasmids expressing 100K protein of HAdV-5 (pHA.h100K.EY) or porcine adenovirus (PAdV)-3 (pHA.p100K.EY) as EYFP fusions proteins, and DsRed fusion proteins. Proteins from the lysates of the cells co-transfected with plasmid DNAs were separated by 12 % SDS-PAGE and probed in Western blot using anti-GFP sera. As expected (Fig. 5.8, panel A), anti-GFP sera detected a 40 kDa major cleavage product and a minor band of

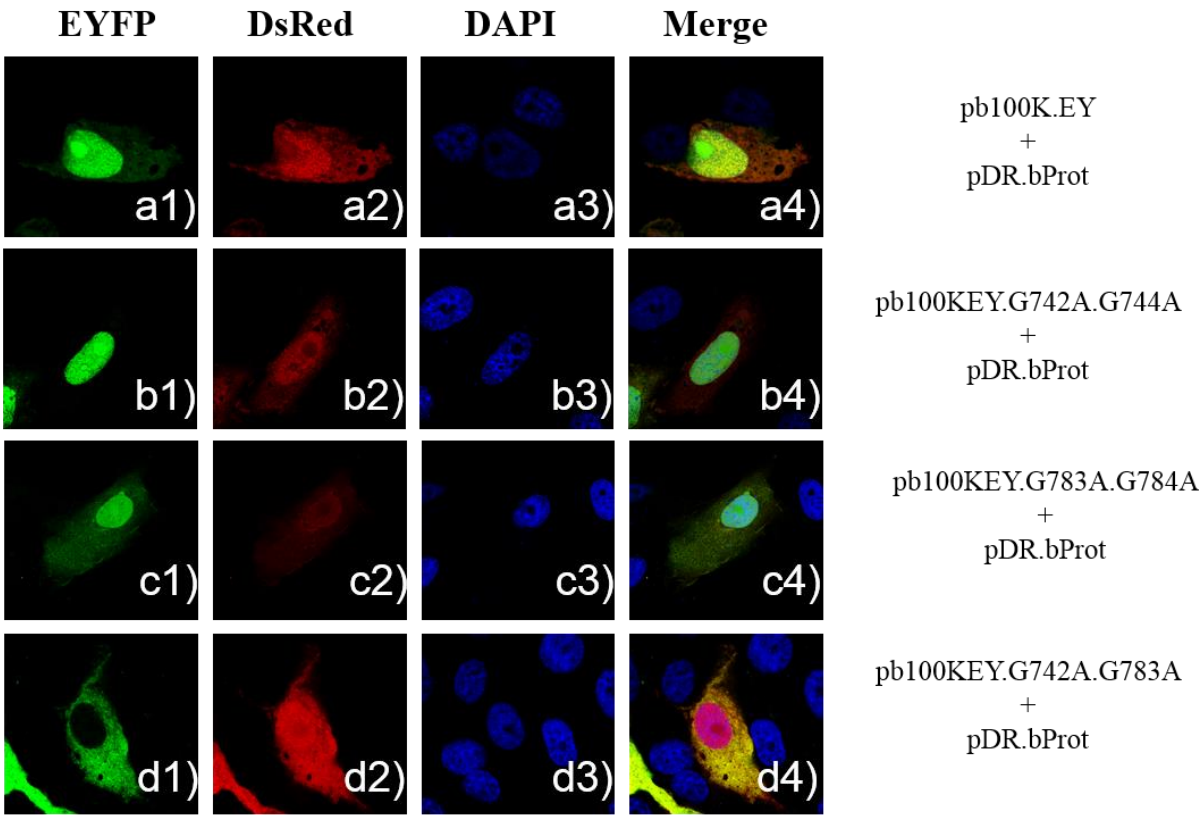

Figure 5.5 Subcellular distribution of BAdV-3 100K cleavage site mutant protein in co-transfected cells. Vero cells cotransfected with indicated plasmid DNAs were analyzed by direct fluorescence at 48 hrs post transfection using Zeiss LSM 5 laser scanning confocal microscope. The name of the plasmid is on the right of the panel.

35 kDa in cells cotransfected with plasmid pHA.b100K.myc.EY + pDR.bProt b) plasmids expressing protease protein of HAdV-5 (pDR.hProt) or PAdV-3 (pDRpProt) as DNAs. No such cleavage product could be detected in cells cotransfected with plasmid pHA.h100K.myc.EY + pDR.hProt or pHA.p100K.myc.EY+pDR.pProt DNAs (Fig. 5.8, panel A). These results are suggestive of the cleavage of 100K protein of BAdV-3 only and not the other adenoviruses in transfection assays.

Secondly, the cells expressing BAdV-3 100K-EYFP fusion protein were co-transfected with plasmid pDR.bProt, pDR.hProt or pDR.pProt DNAs and analysed by Western blot. As seen in figure 5.8 (panel B), proteins of 40 kDa and 36 kDa were detected in cells expressing BAdV-3 100K-EYFP fusion proteins and protease encoded by BAdV-3, HAdV-5, or PAdV-3. No such proteins could be detected in cells expressing BAdV-3 100K-EYFP fusion protein.

5.3.8 Isolation of recombinant BAdV-3 expressing protease cleavage site(s) mutant 100K protein.

In order to determine the role of 100K cleavage in BAdV-3 replication, we generated protease cleavage resistant mutant viruses (Fig. 5.9, panel A). First recombinant virus BAV742 contained substitution of amino acid 742 and 744 from G to A in potential protease cleavage site 1 of 100K. The second recombinant BAV742/783 contained substitution of amino acids 742, 746, 783 and 784 from G to A in potential protease cleavage site 2 of 100K. Introduction of these amino acid substitutions altered the potential protease cleavage site of 100K, which was confirmed by Western blot. As seen in figure 5.9 (panel B), anti-100K antibody (C100) detected a protein of 15 kDa in BAV304a infected cells. No such protein could be detected in cells infected with BAV742 or BAV742/783.

To determine viral gene expression in mutant virus infected cells, the expression of selected

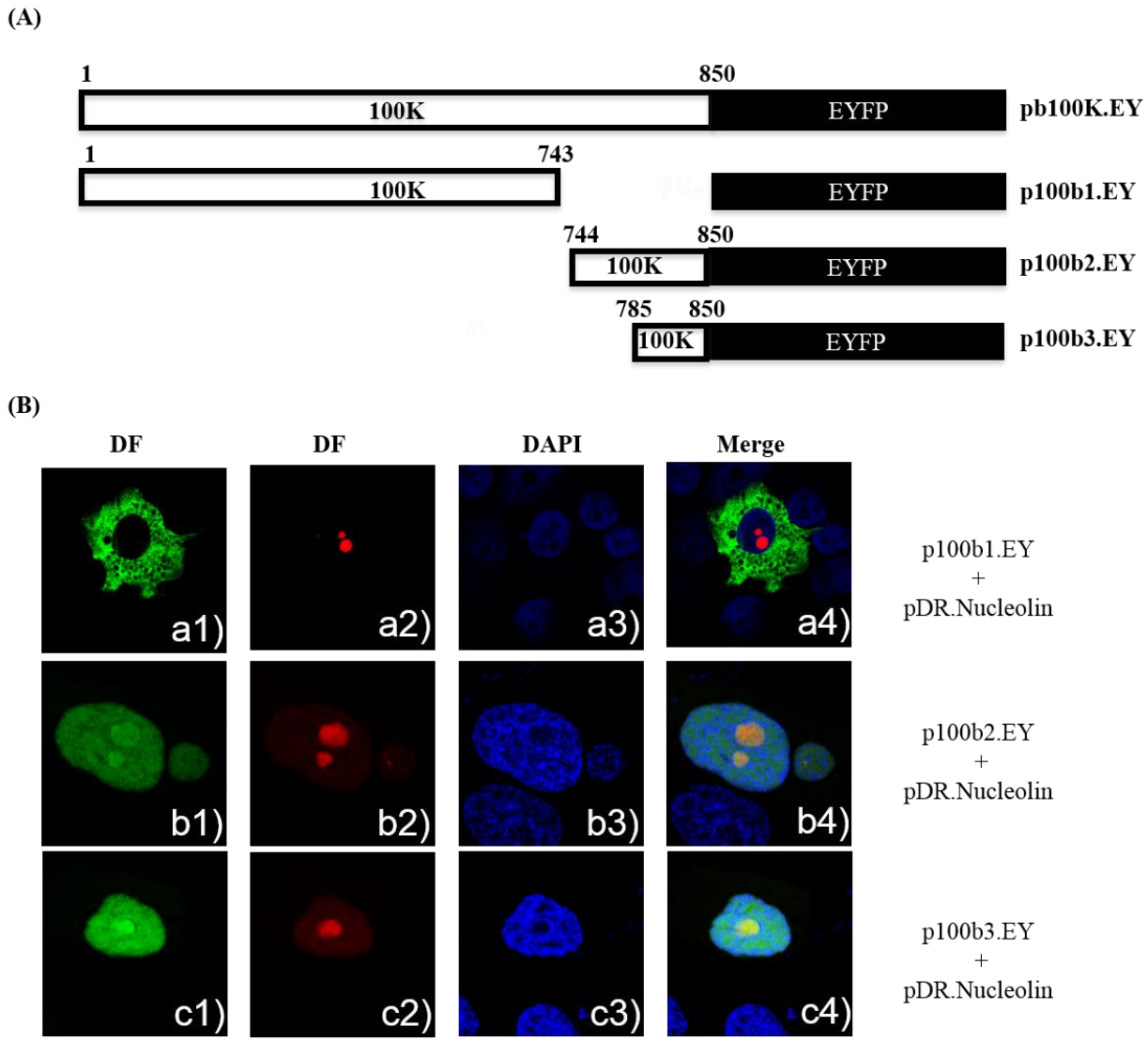

Figure 5.6 Intracellular localization of mutant 100K proteins. (A) Schematic diagram of plasmid DNAs. The origin of DNAs is depicted. The amino acid numbers of 100K are depicted. The name of the plasmid is on the right side of the panel. **(B)** Vero cells co-transfected with indicated plasmid DNAs were analyzed by direct fluorescence at 48 hrs post transfection using Zeiss LSM 5 laser scanning confocal microscope.

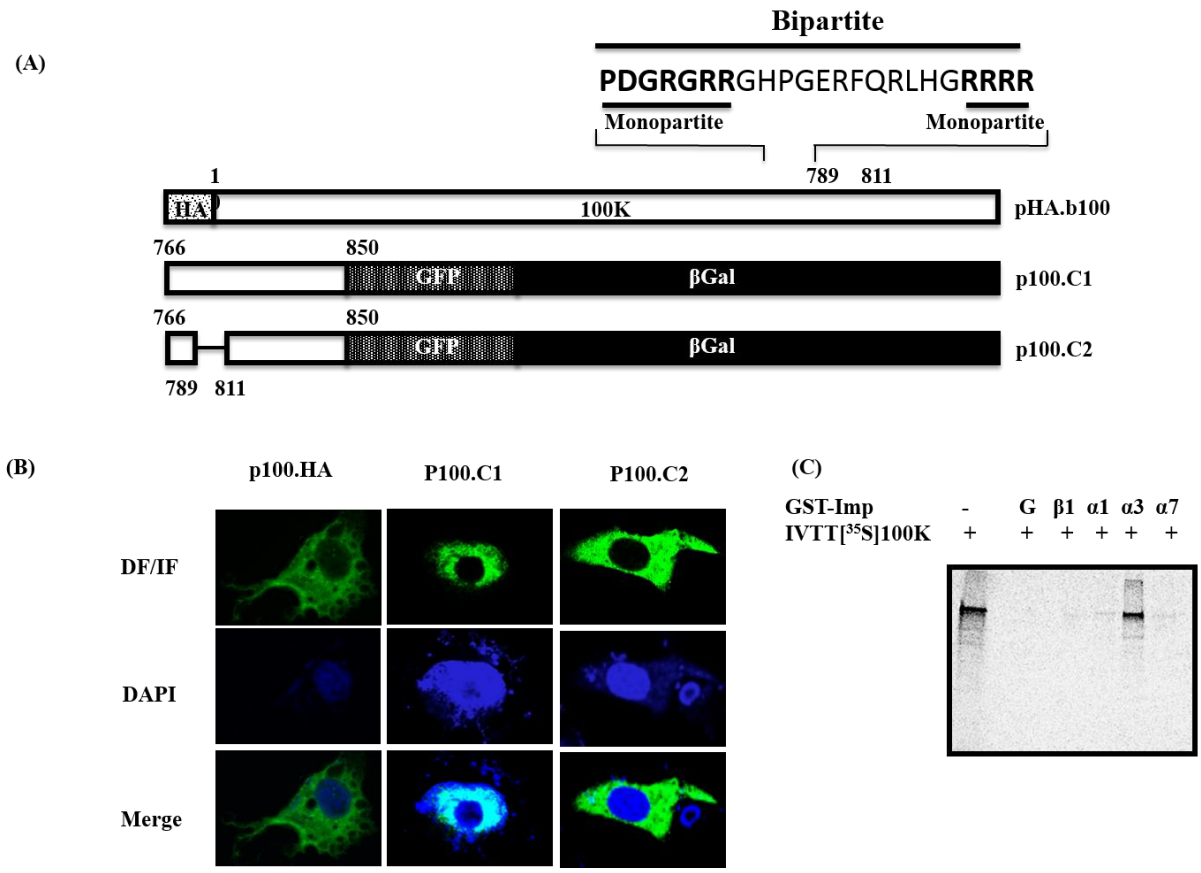

Figure 5.7 Subcellular distribution of nuclear localization signal (NLS) mutant 100K. (A) Schematic diagram of plasmid DNA. The origin of DNAs is depicted. The numbers represent the amino acids of 100K. The amino acid sequence of potential bipartite NLS is depicted. **(B)** Vero cells transfected with indicated plasmid DNAs were analyzed by direct fluorescence microscopy 48 hrs post transfection using Zeiss LSM 5 laser scanning confocal microscope. **(C)** GST pull down of radiolabelled [^{35}S] 100K protein with GST alone or GST importin fusion with β1, α1, α3 and α7.

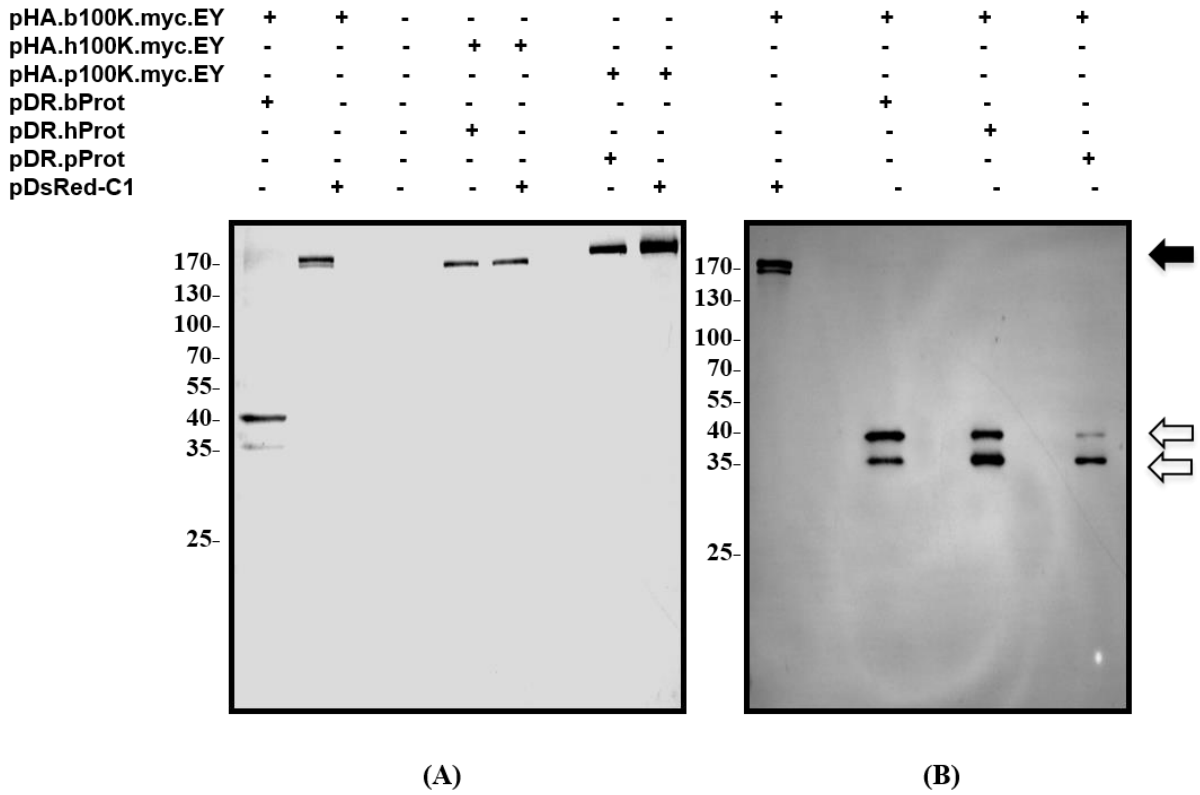

Figure 5.8 Cleavage of adenovirus 100K by different proteases. (A) Proteins from the lysates of 293T cells transfected with indicated plasmid DNAs and harvested at 48 hrs post transfection were separated by 15% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-GFP monoclonal antibody. Filled arrows indicate mature b100K-EY, h100K-EY or p100K-EY fusion protein and empty arrows indicates the cleaved product. **(B)** 293T cells transfected with indicated plasmid DNAs and harvested at 48 hrs post transfection were separated by 15% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-GFP monoclonal antibody. Filled arrows indicate mature b100K-EY fusion protein and empty arrows indicates the cleaved product.

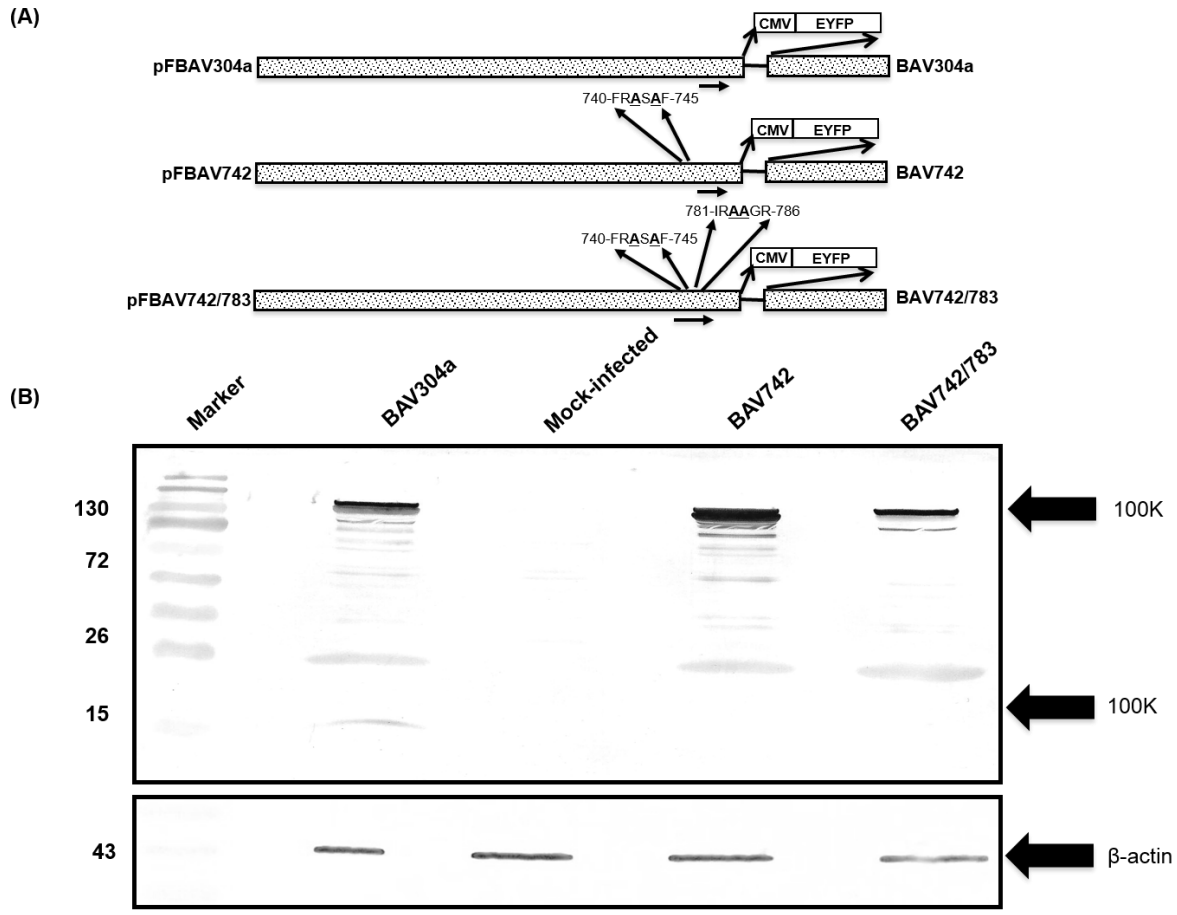

Figure 5.9 Analysis of mutant 100K BAdV-3. (A) Schematic diagram of mutant BAdV-3 genomes. The origin of DNAs is depicted. The horizontal arrows depict the direction of transcription. The name recombinant BAdV-3 is on the right of the panel. The name of the full length plasmid is on the left of the panel. The amino acid number of 100K are depicted along mutant identified protease cleavage sequence. The alanines substituted for glycines are shown in bold and underlined. **(B)** Proteins from the lysates of mock infected or virus infected VIDO DT1 (Du and Tikoo, 2010) cells harvested at 48 hrs post infection were separated by 15% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-100K (C100) sera.

early (DNA binding protein) and late genes (33K) was analyzed by Western blot. As seen in figure 5.10, the 33K protein and DBP were expressed in equal amounts in the wild type and mutant viruses.

5.3.9 Cell associated vs. secreted

To assess whether global cellular protein synthesis is affected during infection with BAV304a and BAV742/783 viruses, cellular protein synthesis assay was performed. The results indicated a similar level of protein synthesis in the BAV304a and BAV-742/783 virus infected cells, suggesting no change in the level of protein synthesis due to mutations of the protease cleavage sites of 100K protein (Fig. 5.11).

To determine if inhibition of cleavage of 100K affected the virus growth, we determined the ability of mutant viruses to grow in MDBK cells. Virus infected cells were harvested at indicated times post infection, freeze thawed five times and the cell lysates were analyzed for virus titer by a $TCID_{50}$ assay. As seen in figure 5.12A, all the three viruses were growing at similar titers at different time points post-infection of MDBK cells. Interestingly, there was a log difference between virus titers between BAV304a and BAV-742/783 (Fig. 5.12B). The results of this assay suggests possible role of cleavage in the release of the BAdV-3 from infected cells.

5.4 DISCUSSION

Production of mature infectious adenovirus particle requires the cleavage of some virus structural proteins by virus specific protease (Diouri et al., 1996). Although cleavage of adenovirus non-structural protein by virus specific recombinant protease has been reported *in vitro* (Ruzindana-Umunyana et al., 2002), no cleavage of an adenovirus non-structural protein by virus protease has been reported *in-vivo.* This may partly be due to the fact that adenovirus protease

appears to be activated only in assembled virions (Ding et al., 1996), which do not contain non-structural proteins (Berk, 2007). Here, we report the cleavage of 100K, a non-structural protein of

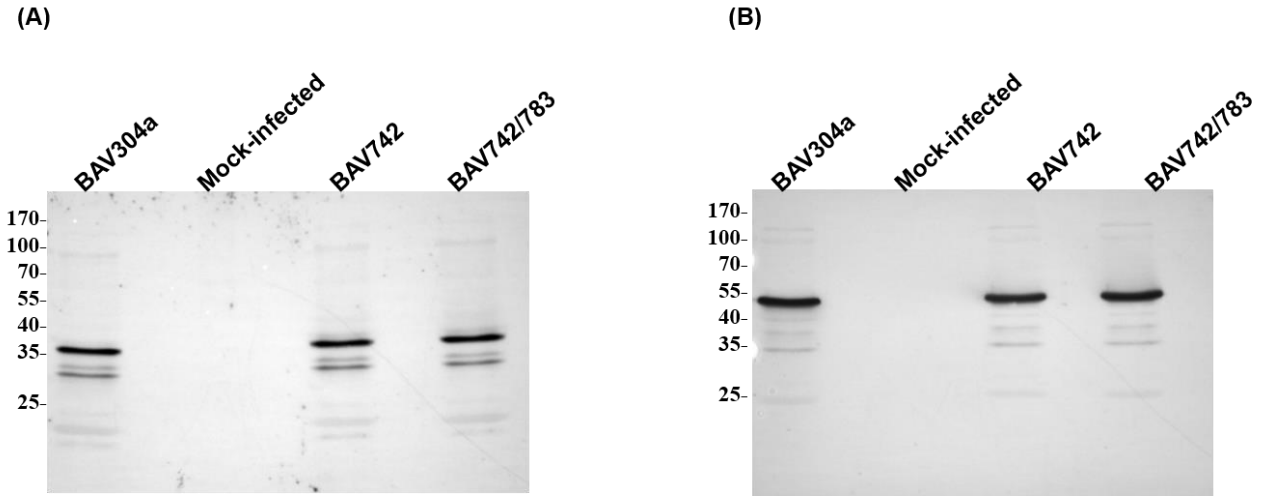

Figure 5.10 Expression of late and early genes in the 100K protease cleavage mutant viruses. Proteins from the lysates of VIDO DT1 cells mock infected or infected with indicated viruses were harvested at 48 h post infection, separated by 15% SDS-PAGE and probed in Western blot using anti-33K antibody (panel A) or anti-DBP serum (panel B) The molecular weight of protein markers in kDa. Are depicted on the left of the panel. Beta-actin was used as the loading control for protein amount.

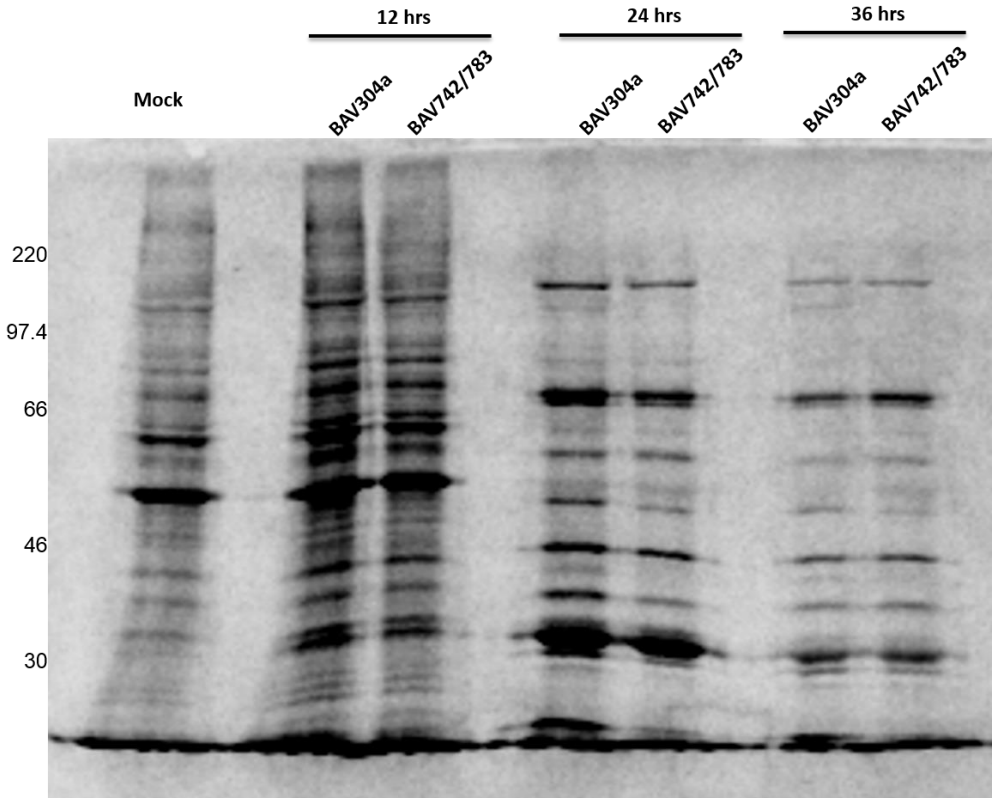

Figure 5.11 Effect of 100K protein cleavage mutant virus on global translation. Confluent monolayers of MDBK cells were infected with mutant or wild-type BAdV-3. At different times post infection, the cells were labelled with radiolabelled methionine for 1 hour. Cells were harvested and analyzed by SDS PAGE and autoradiography for global translation.

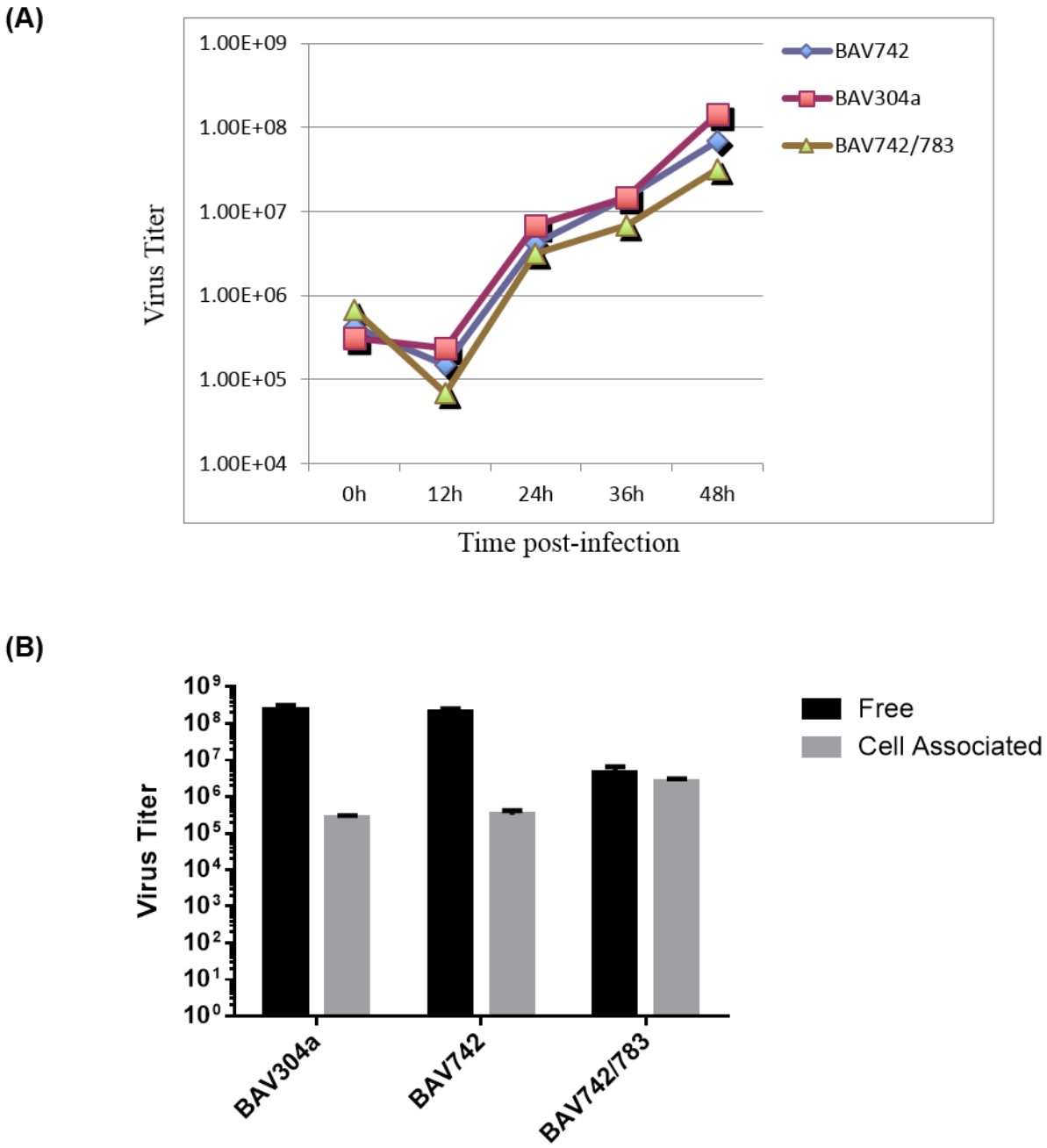

Figure 5.12 Analysis of 100K protein cleavage mutant virus titers. Single step growth curve. Confluent monolayers of MDBK cells were infected with mutant or wild-type BAdV-3. At different times post infection, the cells and the media was collected together **(panel A)** or separated **(panel B)**, freeze thawed and virus was titrated as described (Reddy et al., 1998; Kulshreshtha et al., 2004).

bovine adenovirus-3 by viral protease in transfected and BAdV-3 infected cells. Moreover, we demonstrate that cleavage of BAdV-3 100K appears essential for nuclear localization of 100K but not for efficient virus replication. Our study suggests the need for investigation in the protease activation mechanism.

The 100K protein of BAdV-3 localizes to the nucleus in infected cells. Analysis of 100K protein predicted a bipartite nuclear localization at amino acids 789-811, which is sufficient to localize GFPβGal fusion protein (Wu et al., 2004) to the nucleus in transfected cells. However, BAdV-3 100K protein localized to cytoplasm in transfected cells suggesting that potential NLS is not functional when 100K is expressed alone in transfected cell. In contrast, 100K of HAdV-2/5 localizes to nucleus in transfected cells (Cuesta et al., 2004; Koyuncu and Dobner, 2009). Interestingly, co expression of 100K with protease but not hexon localized 100K in the nucleus of transfected cells. Although pVI (Wodrich et al., 2003) or 100K (Hong et al., 2005) of adenovirus has been reported to be required for partial nuclear localization of hexon, there appears no report suggesting the requirement of adenovirus protease for nuclear transport of an adenovirus protein.

Recent studies have demonstrated significant variation in the structure, sub cellular localization and function of adenovirus protein homologs encoded by different members of *Mastadenoviruses* (Stracker et al., 2005; Blanchette et al., 2013; Cheng et al., 2013). Several lines of evidence suggest significant differences may exist in 100K encoded by different *Mastadenoviruses.* First**,** analysis of amino acid sequences identified two consensus protease cleavage sites (amino acid 740-745 and amino acid 781-786) in 100K protein encoded by BAdV-3 but not in 100K protein encoded by HAdV-5 (Fig. 5.8; panel A) or PAdV-3 (Fig. 5.8; panel A). Secondly, the protease encoded by BAdV-3 can cleave BAdV-3 100K protein in transfected cells. Moreover, cleavage of BAdV-3 100K protein could be detected in infected cells. Thirdly, cleavage

of HAdV-5 or PAdV-3 100K proteins by proteases encoded by HAdV-5 or BAdV-3 could not be detected in transfected cells. Fourthly, proteases encoded by HAdV-5 or PAdV-3 also cleave BAdV-3 100K in transfected cells. Finally, BAdV-3 100K cleavage appears specific as cleavage of mutant 100K containing altered protease cleavage sites could not be detected in transfected cells co-expressing protease.

The current model of adenovirus protease activation suggest the requirement of two viral cofactors namely C-terminal 11 amino acids of pVI and adenoviral DNA (Mangel et al., 1993). Adenoviral protease is synthesized in the inactive form and can only be activated in the presence of cofactors in the nucleus of the adenovirus infected cells (Mangel et al., 2003). Our findings suggest the important aspect of adenoviral protease activation in the absence of cofactors and in the co-transfected cells. Interestingly, BAdV-3 100K can be cleaved by HAdV-5 (Fig. 5.8B) and PAdV-3 (Fig. 5.8B) proteases but the efficiency varies for different sites among proteases. Cleavage of BAdV-3 100K protein by protease in the plasmid cotransfected cells can serve as the basis for further studies.

Although BAdV-3 100K protein alone is predominantly localized in the cytoplasm of the transfected cells, the co-expression with protease localizes 100K predominantly to the nucleus of the transfected cells. Further analysis suggest that C-terminal protease cleaved fragments of 100K are predominantly localized in the nucleus of the transfected cells co-expressing protease with wild type 100K or single protease cleavage site mutant 100K but not with double protease cleavage site mutant of 100K-EYFP fusion protein. It is possible that C-terminal cleavage fragment of 100K-EYFP fusion protein is passively diffused into the nucleus. Alternatively, it is possible that C-terminal cleavage fragments contain nuclear localization signal(s) and are actively transported to the nucleus using importin α/β import pathway. Support for this comes from the fact that a)

analysis of the protein sequence identified a bipartite NLS (amino acid 789-811; figure5.7B) in C-terminal region of BAdV-3 100K protein, b) amino acid 744-850 were sufficient to direct the nuclear import of a predominantly cytoplasmic fusion protein (GFPβGal), c) amino acid 744-850 containing deletion of amino acid 899-811 were not sufficient to direct the nuclear import of a predominantly cytoplasmic fusion protein (GFPβGal). Surprisingly, despite the presence of NLS, 100K is not transported to the nucleus in transfected cells. It is possible that other viral protein (s) may be required for transport of 100K to nucleus. However, co-expression of 100K with other BAdV-3 late proteins except protease does not alter the localization of 100K. Alternatively, it is possible that 100K protein is retained in the cytoplasm by interacting with a cellular protein. Interestingly, the central region (amino acid 498-588) of 100K interacts with cytoplasmic dynein light chain Tctex 1 (DYNLT1) protein (Chapter 4). Interaction of viral structural proteins including adenovirus hexon with cytoplasmic dynein mediates transport of the virus across the cytoplasm of infected cells (Bremner et al., 2009). Since 100K is a non-structural protein, we hypothesize that interaction of 100K protein with DYNLT1 helps to retain 100K in the cytoplasm to perform an important function presumably the inhibition of cellular mRNA translation (Cuesta et al., 2000) and enhancement of adenovirus late mRNA translation (Xi et al., 2004). It is interesting to note that a) amino acid 1-726 of 100K are required for regulating cellular and late adenoviral mRNA translation (Cuesta et al., 2004; Xi et al., 2004) and b) both regulation of translation (Zhang et al., 1994) and cleavage of BAdV-3 100K occur at late phase of adenovirus infection. Interaction of HAdV-2 E3 nonstructural protein 19K with components of microtubules has been suggested to retain class I major histocompatibility complex molecules when complexed with gp19 into endoplasmic reticulum thus down regulating cell surface expression of MHC-1 proteins (Dahllof et al., 1991).

The significance of our finding about cleavage of 100K in BAdV-3 infection and localization of C-terminal cleavage fragment(s) to nucleus is not clear. Our results suggest that cleavage of 100K at amino acid 742 and 783 is not essential for efficient replication and generation of progeny BAdV-3. Since the cleavage is detected at late times post BAdV-3 infection, we hypothesized that it may have a role in the events occurring late in BAdV-3 infection including altered ribosomal RNA processing (Paterson, 2010), inhibition of cellular mRNA translation (Lisanework and Tikoo, manuscript in preparation), or release of virus. However, there was no difference in ribosomal RNA processing or cellular mRNA translation in cells infected with wild type or mutant BAdV-3 expressing protease cleavage resistant 100K (data not shown). In contrast, although WT and protease cleavage mutant BAdV-3s grew to similar titers, we observed one log less virus in the supernatant of cells infected with protease cleavage mutant compared to wild type BAdV-3. It is possible that nuclear localization of C-terminal cleavage fragment of 100K may help in lysis of infected cells helping in the release of progeny virus in media. The cleavage of 100K protein in BAdV-3 infected cells is unique event and appear to be absent in the HAdV-5 and PAdV-3 infection process.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

Earlier reports indicate that adenovirus 100K protein is involved a) in enhancing the translation of late adenoviral mRNAs by ribosomal shunting mechanism, b) in inhibiting the translation of capped cellular mRNAs, and c) in trimerization of adenovirus hexon protein (Cuesta et al., 2000; Hong et al., 2005; Xi et al., 2005). Some of the identified functions require interaction of adenoviral 100K protein with cellular (Xi et al., 2005) and viral protein(s) (Hong et al., 2005; Kulshreshtha and Tikoo, 2008). Although, the members of the *Adenoviridae* family encode the homologs of non-structural protein 100K with similar genomic organization (Davison et al., 2003), the proteins sequences have shown variable (27.6 % to 52.1%) homologies (Reddy et al., 1998). Since significant differences have been reported to exist in structure and function of adenovirus protein homologs showing significant homology (Blanchette et al., 2013; Cheng et al., 2013; ; Stracker et al., 2005), we sought to analyze BAdV-3 100K protein in detail. Thus, the primary aim of this work was to characterize 100K protein and elucidate its interaction with other viral/ cellular proteins.

Using antisera raised in rabbits against peptides representing 100K protein (N terminus or C-terminus), we detected a protein of 130 kDa at 24 to 48 hrs post-infection. Interestingly, 130 kDa protein could also be detected as a doublet at 36 and 48 hrs post-infection. Presence of an extra band, also reported in HAdV-2 infected cells (Hong et al., 2005) at later time point post-infection is suggestive of the post-translational modification like methylation (Koyuncu and Dobner, 2009) or phosphorylation (Xi et al., 2005) of the 100K protein.

Like HAdV-5 (Koyuncu and Dobner, 2009), the 100K protein is localized to the nucleus and cytoplasm of BAdV-3 infected cells. However, unlike HAdV-5 100K protein (Koyuncu and Dobner, 2009), BAdV-3 100K-EYFP fusion protein localized exclusively to the cytoplasm of

transfected cells. Interestingly, 100K-EYFP fusion protein localized to nucleus of BAdV-3 infected cells suggesting that nuclear localization of 100K may require other viral or cellular proteins in infected/transfected cells. Although viral proteins known to interact with adenoviral 100K did not affect the localization of 100K protein, surprisingly co-expression of 100K with adenoviral protease localized BAdV-3 100K protein to the nucleus in co-transfected cells. Analysis of transfected cells revealed that protease cleaves 100K protein at two consensus protease cleavage sites and only C-terminal cleaved fragments of 100K containing bipartite nuclear localization signals are localized to nucleus.

Like HAdV-5 (Cuesta et al., 2000; Xi et al., 2005), the cellular mRNA translation (Ayalew and Tikoo, unpublished results) and ribosomal processing (Paterson, 2010) is inhibited at late times post BAdV-3 infection. Interestingly, the cleavage of 100K also appears to occur at late times (36-48 hrs) post BAdV-3 infection. However, analysis of recombinant BAdV-3 expressing protease cleavage mutant suggested that cleavage of 100K does not appear to be essential for the replication of BAdV-3 or events observed at late times post BAdV-3 infection including inhibition of cellular mRNA translation and ribosomal processing. Nevertheless, though protease cleavage mutant grew to similar titers to WT BAdV-3, less virus (1 log) was observed in supernatant of protease cleavage mutant infected cells than in supernatant of Wt BAdV-3 infected cells. It is possible that nuclear localization of C-terminal cleavage fragment of 100K may help in lysis of infected cells helping in the release of progeny virus in media. Virus release from the infected cells is the results of the cleavage of cytokeratin 18 by viral protease (Chen et al., 1993), action of ADP (Tollefson et al., 1996) and interaction of accumulated fiber with CAR (Walters et al., 2002).

Protein-protein interactions have been reported in adenovirus infected cells (Wickham et al., 1993; Berk, 2005; Bremner et al., 2009; Arnberg. 2012). The involvement of 100K protein in

different steps of adenovirus replication (Cepko and Sharp, 1982; Cuesta et al., 2000; Hong et al., 2005; Xi et al., 2005) may require protein-protein interactions. Like other adenovirus proteins, adenovirus 100K protein is reported to interact with hexon (Frost and Williams, 1978; Cepko and Sharp, 1982). Using several assays (yeast two-hybrid, GST pull-down and co-immunoprecipitation assays), earlier our laboratory demonstrated that 100K interacts with 33K protein in BAdV-3 infected cells (Kulshreshtha and Tikoo, 2008). We reconfirmed these results using bimolecular fluorescence complementation assays (BiFC). Using mutant BAdV-3 100K proteins, we demonstrate that the 33K protein interacting region of 100K appears to be localized between amino acids 624-637. Several attempts to isolate mutant BAdV-3 expressing mutant 100K containing deletion of 33K protein interacting region proved futile suggesting the essential role of this interaction (100K-33K) or interacting region (AA 624-637) of 100K protein in the viral life cycle. It is also possible that deletion of 13 amino acid interacting region of 100K might alter the 100K folding and/or other unidentified function of 100K. Further experiments are in progress to isolate and analyze recombinant BAdV-3 expressing mutant 100K (containing point mutation/substitution point mutation) protein.

7.0 References

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